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Stenotrophomonas maltophilia in patients with bronchiectasis

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Own work declaration

I, Elena PEREZ FERNANDEZ, certify that:

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- (b) the work is my own, and
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Abstract

Bronchiectasis is an acquired irreversible bronchopulmonary disease where bronchial walls in the lungs are permanently inflamed and dilated. This leads to a 'vicious cycle' between chronic bacterial infection and immune dysregulation. One of the bacterial infections patients with bronchiectasis experience is *S. maltophilia*, a Gram-negative gamma proteobacteria with intrinsic multidrug resistance and widely found in the environment. *S. maltophilia* is also a common infection in CF patients but has poorly been characterised in bronchiectasis. This study looks at its significance in bronchiectasis and tries to establish a comparison between environmental and clinical strains as well as to elucidate the question of its pathogenicity which is still controversial.

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1. Introduction

1.1. Bronchiectasis

Bronchiectasis is an acquired irreversible bronchopulmonary disease where the bronchial wall in the lungs is permanently inflamed and dilated (José and Brown, 2014). This leads to what is sometimes referred to as a 'vicious cycle' (Cole, 1986) between chronic bacterial infection and immune dysregulation (Fig. 1.1): the damaged function of the bronchi results in an accumulation of mucus in the lungs which is conducive to bacterial infections which, in turn, cause further damage to the bronchial wall of the lung by eliciting an inflammatory response and further debilitate the mucociliary apparatus which loses its ability to properly clear mucus from the lungs (Boyton, 2012).

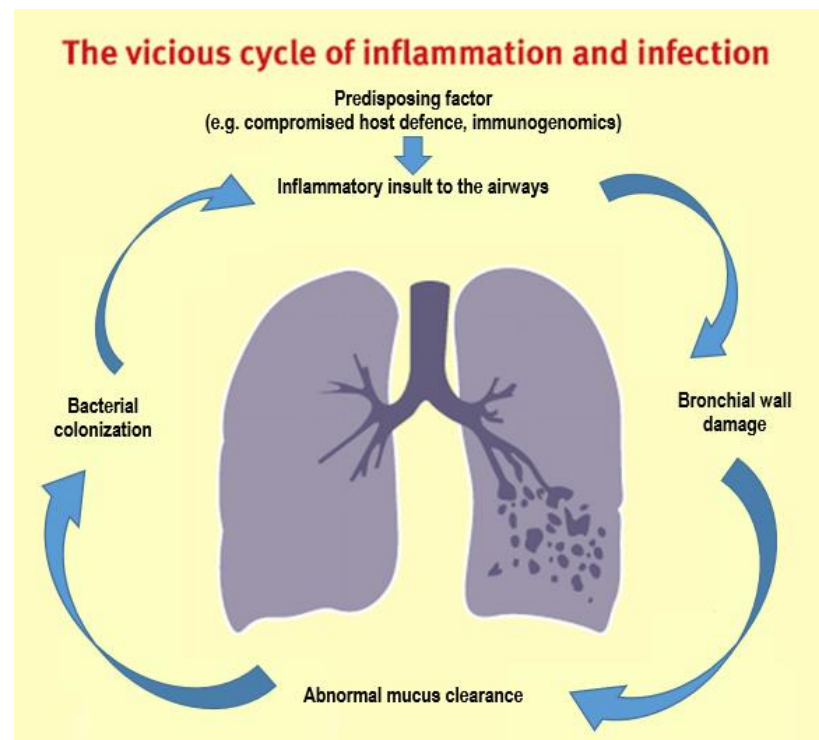


Figure 1.1 The vicious cycle of inflammation and infection in bronchiectasis (adapted from José and Brown, 2014).

1.1.1. Aetiology of bronchiectasis

Many cases of bronchiectasis arise without any known cause and are referred to as *idiopathic*. However, when a cause is found, the most common aetiology is past infection such as pneumonia, tuberculosis and viral infections (Hacken and van der Molen, 2010).

Findings from several epidemiology studies demonstrated that some specific ethnic populations have a significantly higher incidence of bronchiectasis than others: for example, a study cohort from central Australia showed a 40-fold increase in the rate of bronchiectasis among Aboriginal children compared to the rate of their non-Indigenous counterparts (Chang *et al.*, 2002). This evidence suggests that risk populations may also have underlying immuno-genetics predisposing them for the disorder.

1.1.2. Clinical features and diagnosis

The main clinical features that can help detection of bronchiectasis are chronic cough, production of sputum and persistent bacterial infection although some patients presenting mild forms of the disease can be asymptomatic (Boyton *et al.*, 2013). Those symptoms also overlap with other pulmonary diseases with a higher incidence than bronchiectasis such as asthma, tracheobronchial infection and chronic obstructive pulmonary disease (COPD) which make it difficult to diagnose (Hacken and van der Molen, 2010). High resolution computed tomography (HRCT) of the chest (Figure 1.2) is currently the gold standard for diagnosis (Boyton, 2012).

Following the HRCT, a score ranging from 1-18 is attributed to the patient depending on the number of lobes affected and their degree of dilatation (Chalmers *et al.*, 2014). With the widespread use of CT scans, figures for incidence of bronchiectasis per year are on the increase in recent years, suggesting it might have been significantly underdiagnosed in the past (King, 2011). In the United Kingdom, for instance, it is estimated that over 1 in 1000 people currently live with bronchiectasis (Hill, 2012) and a recent cohort study from the UK found that incidence of bronchiectasis in women had increased from 21.2 to 35.2 and in men from 18.2 to 26.9 per 100 000 per person-years from 2004 to 2013 (Quint *et al.*, 2016).

However, the scope of severity of the condition varies greatly between patients and the HRCT score does not provide an accurate way for assessing it as it misses many other factors that can also influence the severity. Hence, in addition to the radiological diagnosis by HRCT, clinicians use two scoring systems: the Bronchiectasis Severity Index (BSI) (Chalmers *et al.*, 2014) and the FACED score (Martínez-García *et al.*, 2014; Guan, Chen and Zhong, 2016).

The BSI is an algorithm which was designed to comprehensively assess the severity of the condition for each patient and provide a prediction of future exacerbations and mortality (Chalmers *et al.*, 2014) (Table 1.1). This takes into account the following parameters: age, BMI (Body Mass Index), spirometry values (% FEV₁ predicted),

previous hospital admissions, exacerbation(s) frequency, presence of *Pseudomonas aeruginosa* or other bacteria, the radiological severity (i.e. number of lobes affected) and the medical research council (MRC) breathlessness score. An online calculator is available via the following website: <http://www.bronchiectasisseverity.com/> where clinicians just need to insert the patient's values for each of the parameters and the score is automatically calculated: a score between 0 and 4 corresponds to a mild form of bronchiectasis; a score between 5 and 8 suggests a moderate stage of bronchiectasis and finally, a score equal to or above 9 relates to severe cases of bronchiectasis (Chalmers *et al.*, 2014).

Table 1.1 Rates of mortality and hospitalisation over 1- and 4-years follow-up based on the bronchiectasis severity score (BSI)

| Severity | Score range | 1 year | | 4 years | |
|----------|-------------|-------------------|-------------------------|-------------------|-------------------------|
| | | Rate of mortality | Rate of hospitalisation | Rate of mortality | Rate of hospitalisation |
| Mild | 0-4 | 0-2.8% | 0-3.4% | 0-5.3% | 0-9.2% |
| Moderate | 5-8 | 0.8-4.8% | 1.0-7.2% | 4-11.3% | 9.9-19.4% |
| Severe | >9 | 7.6-10.5% | 16.7-52.6% | 9.9-29.2% | 41.2-80.4% |

On the other hand, the FACED score is a 7 points grading system taking into account five dichotomised parameters: the FEV₁ score, the age, *Pseudomonas aeruginosa* chronic colonisation, the extent of bronchiectasis and the level of dyspnoea based on the Medical Research Council (MRC) scale (Table 1.2) and which provides a prediction of 5-year all-cause mortality rate for each severity category (Martínez-García *et al.*, 2014).

Table 1.2 Scoring system of the five dichotomised parameters included in the FACED score

| | | | |
|--------------------------------------|--|--------------------------------|-------------------------------|
| F | FEV ₁ | ≥ 50 % = 0 points | < 50 % = 2 points |
| A | Age | > 70 years = 0 points | ≥ 70 years = 2 points |
| C | Chronic colonisation with <i>P. aeruginosa</i> | No = 0 points | Yes = 1 point |
| E | Extent of bronchiectasis | 1-2 lobe(s) affected = 1 point | ≥ 2 lobes affected = 2 points |
| D | Dyspnoea MRC score | 0-II = 0 points | III-IV = 1 point |
| TOTAL SCORE : | | 5-YEAR MORTALITY RATE : | |
| • 0-2 points MILD BRONCHIECTASIS | | 4% | |
| • 3-4 points MODERATE BRONCHIECTASIS | | 25% | |
| • 5-7 points SEVERE BRONCHIECTASIS | | 56% | |

This classification may allow for more personalised treatment and for deciding on the best treatment after evaluating the risk/benefit ratio (Chalmers *et al.*, 2014).

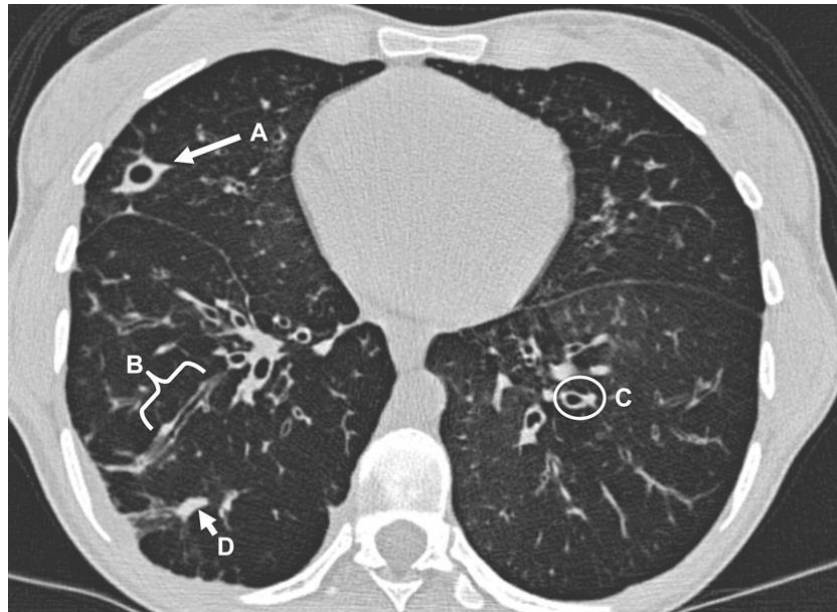


Figure 1.2 HRCT scan showing the main signs of bronchiectasis. A shows a bronchus terminating in a cyst; B shows the lack of bronchial tapering as it travels to the periphery of the lung; C shows the sign of a signet ring where the bronchus is larger than the accompanying vessel; D shows a mucus plug, completely blocking the airway lumen (McShane *et al.*, 2013).

1.1.3. Treatment and management

Bronchiectasis is a progressive disease; its severity increases with time in patients with more advanced bronchiectasis. However, the aim with early detection is to slow down the progression and help manage the disease better so that, following an appropriate treatment plan, prognosis can be improved and life expectancy and quality of life (QoL) maintained (Hill, 2012).

Due to the impaired mucociliary clearance function, the mainstay of bronchiectasis management is chest physiotherapy (Fig. 1.3): all patients, regardless of the severity of their condition are encouraged to learn and practice airway clearance techniques in order to facilitate sputum expectoration (Chalmers, Aliberti and Blasi, 2015). The most common techniques for doing so are active cycle breathing technique, autogenic drainage and the use of positive expiratory pressure devices (Snijders *et al.*, 2015).

To tackle bacterial colonisation and airway inflammation involved in bronchiectasis, the most common treatments consist of short- or long-term inhaled antibiotics, inhaled corticosteroids and macrolides (Fig 1.3).

Most commonly used macrolides are azithromycin (Wong *et al.*, 2012) and erythromycin (Serisier *et al.*, 2013; Chalmers *et al.*, 2015). Recent studies provide evidence for their beneficial effects in bronchiectasis patients where they have been proven to significantly reduce the rate of exacerbations and a meta-analysis of randomised controlled trials (Fan *et al.*, 2015) suggested that they improve the St George Respiratory Questionnaire (SGRQ) score –a disease-specific questionnaire on QoL of patients (Wilson *et al.*, 1997). However, the use of macrolides has also been associated with important side-effects on the gastrointestinal and cardiovascular systems, increased antimicrobial resistance, potential risk of hearing loss and potential predisposition to environmental mycobacteria infection which can lead to macrolide resistance (Fan *et al.*, 2015).

On the other hand, the use of inhaled corticosteroids, regular high dose inhaled steroid treatment was shown to result in a reduced sputum volume and to decrease the amount of inflammatory markers found in sputum, also resulting in an improvement of QoL (SGRQ score). However, there is no clear evidence for improvement of patient's lung function or rate of exacerbations and the BTS guidelines do not support the routine use of long-term inhaled corticosteroids (Goyal and Chang, 2014). Importantly, reported cases of adrenal suppression and increased risk of pneumonia in COPD patients associated with inhaled steroids treatment are raising serious concerns (Holme *et al.*, 2008).

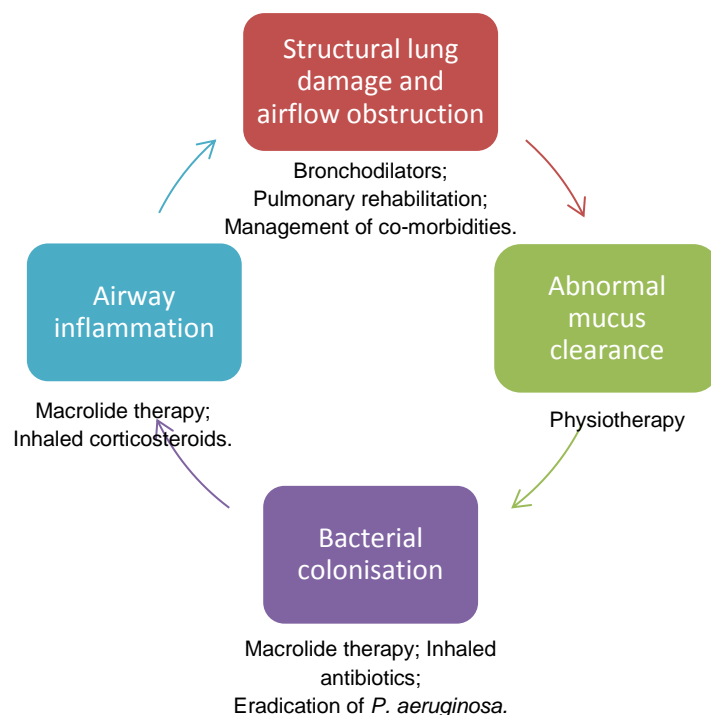


Figure 1.3 Current recommended therapies for each of the elements of Cole's vicious cycle of inflammation and infection (adapted from Chalmers *et al.* 2015).

Finally, the advantage of inhaled antibiotics is their ability to deliver higher concentrations of antibiotics to the airways while simultaneously reducing the potential side effects inherent to the systemic absorption occurring with oral or intravenous (IV) delivery (Brodt, Stovold and Zhang, 2014).

Most widely used inhaled antibiotics generally focus on treating chronic infection with *P. aeruginosa* by colomycin, tobramycin and gentamicin (Chalmers *et al.*, 2015). It is worth noting that these antibiotics have also been reported to elicit significant problems of tolerability among patients and further trials are needed to determine whether the benefits outweigh the potential complications involved with their use in patients with bronchiectasis.

To sum up, all these treatments always come at a risk of promoting antibiotic resistance and side effects and therefore, it is important to stress the need for therapy to be tailored to each patient and only intensified when necessary and make sure the side effects do not outweigh the intended benefits of the therapy. In addition, in the long term, limiting of antibiotic prescriptions will also result in a reduction of the healthcare spending (Chalmers *et al.*, 2012).

1.1.4. Propensity for infections

As mentioned previously, patients with bronchiectasis are prone to infections: more than two thirds of patients with bronchiectasis are chronically colonised with bacteria (Hill, 2012). An interesting hypothesis is that, due to the “vicious cycle of inflammation and infection”, resulting antibiotic therapy disrupts the lung microbiome leading to a simplified microbiota with some empty niches that leave room for further establishment of microbial pathogens (Boyton, 2012). Some of the most common colonising organisms in bronchiectasis are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (King, 2011). These are opportunistic pathogens and all share the selective ability to adhere to the epithelium in the airway and produce biofilms that protect them from immune components stimulated by their presence (King, 2011). Another less common bacterial infection also reported in events of exacerbations in bronchiectasis is *Stenotrophomonas maltophilia* (Boyton, 2012).

1.2. *Stenotrophomonas maltophilia*

S. maltophilia is not found in the human natural flora but it is commonly found as an opportunistic pathogen in immunocompromised patients (Denton and Kerr, 1998). One of the first reported cases of *S. maltophilia* in bronchiectasis was from a study case from 1993 where the mucoid version of the opportunistic pathogen was found to be the cause of a pneumonia in a bronchiectasis patient (Irifune *et al.*, 1994). Unlike the more common pathogens previously mentioned, nothing has yet been published about the role of *S. maltophilia* in bronchiectasis and little is known about its effects on the disorder nor the causes for infection.

1.2.1. Classification

S. maltophilia was first attributed its own genus in 1993 (Palleroni and Bradbury, 1993) after being characterised initially as a member of the *Pseudomonas* genus by Hugh and Ryschenkow (1961) and later controversially moved to the genus *Xanthomonas* mainly based on segmental DNA homology by Swings *et al.* (1983).

S. maltophilia is an aerobic non-fermenting and generally oxidase negative, Gram-negative bacillus from the gamma proteobacteria class (Crossman *et al.*, 2008).

The genus *Stenotrophomonas* is currently composed of eight different species (*S. maltophilia*, *S. nitrireducens*, *S. acidaminiphila*, *S. rhizophila*, *S. koreensis*, *S. chelatiphaga*, *S. terrae* and *S. humi*) which have been determined following phenotypic and genotypic analysis. All species are widely found in the environment where they seem to hold a leading role in nitrogen and sulphur cycles from which many plants benefit (Ryan *et al.*, 2009).

The particularity of the *S. maltophilia* species is that it is the only species from the genus known to be pathogenic to humans (Ryan *et al.*, 2009). It was initially only considered as a commensal but there is increasing evidence to show that it can also be a *true* pathogen. Its infection can originate in a variety of tissues and lead to serious complications, bacteraemia being the most common. It is usually caused by contaminated intravascular devices, ultimately leading to septicaemia with a high mortality rate (Denton and Kerr, 1998). It is worth noting that the respiratory tract remains the principal site of isolation of *S. maltophilia* from patients and accounts for the origin of 56 to 69% of total isolates. Lungs usually become infected through the use of contaminated nebulizers which can subsequently cause pneumonia (Denton and Kerr, 1998).

1.2.2. Intrinsic multidrug resistance

S. maltophilia is characterised by intrinsic drug resistance to a broad array of antibiotics such as macrolides, β -lactams, aminoglycosides and quinolones (Brooke, 2012). Evidence suggests this multidrug resistance is mainly conferred by efflux pumps; a comparative genomic analysis of strains from both environmental and clinical origin for which full genome sequences are available identified around 50 genes involved in multidrug resistance in each of the strains, of which about 40 to 50% are operons for efflux pumps (Younou *et al.*, 2015).

From, the same study, it was observed that environmental strains often carried a superior amount of efflux pumps than the clinical strains. This supports the fact that these pumps most likely play additional roles related to the natural habitat of *S. maltophilia* such as detoxification of heavy metals and solvents, trafficking of quorum-sensing molecules, providing it with valuable assets to compete against other bacteria sharing its ecological niche (Younou *et al.*, 2015).

Thus, it seems that no antibiotic or anthropic selective pressures were required for *S. maltophilia* to develop its intrinsic antimicrobial resistance but it is rather an outcome of the natural selection for its beneficial properties in its natural habitat (Martínez, 2008). Furthermore, Younou *et al.* (2015) also revealed in their study a high functional redundancy in the genes coding for efflux pumps since the lack of one did not necessarily correspond to a loss of its function.

1.2.3. Genomic variation

Comparative analysis of the available sequences allowed to define the core genome of *S. maltophilia* as being composed of 1,647 conserved proteins, the remaining 55 to 65% of proteins forming the accessory genome (Younou *et al.*, 2015). This provided further evidence that a significant part of *S. maltophilia* antimicrobial resistance is not clonal but acquired, probably through horizontal gene transfer. This illustrates the tremendous level of genomic variation in *S. maltophilia*.

1.2.4. Biofilm formation

S. maltophilia is quite ubiquitous in the environment as a waterborne bacteria and has been isolated from roots, soil, wastewater plants and salad (Qureshi *et al.*, 2005). As a pathogen, another feature that confers it a selective advantage to easily grow in hospital settings is its high production of appendages such as fimbriae and pili. These explain the remarkable ability of *S. maltophilia* to form biofilms and allow it to adhere to plastic surfaces on indwelling devices from which it can easily reach an immunocompromised host organism in which to settle (Denton *et al.*, 2003). In

such cases, infection can generally be treated by removal or replacement of the contaminated device.

1.2.5. Virulence

S. maltophilia is not very virulent, there is no evidence of patient-to-patient contamination and, even within wards, patients infected carry different –and often multiple– strains (Brooke, 2012). In fact, many studies have shown that clinical strains of *S. maltophilia* have a higher frequency of mutation than strains isolated from the environment; they can easily adapt to the local environment of their host (Berg, Roskot and Smalla, 1999). The only instances of community outbreaks reported have been as a result of infection from direct contact with ICU healthcare workers carrying the bacteria on their hands (Schable *et al.*, 1991).

With their great capacity to adhere to surfaces by forming biofilms (Guyot, Turton and Garner, 2013), an easy step to prevent contamination of immunocompromised people is to carefully disinfect drinkable water distribution systems using 3% hydrogen peroxide which was proved to be efficient against *S. maltophilia* (Sacchetti, De Luca and Zanetti, 2009). However, such route of transmission is not likely to be a significant threat of contamination since studies have been unable to show a correlation between strains of *S. maltophilia* present in the water from hospital environments with the pathogenic strains isolated from patients (Marzuillo *et al.*, 2009). The most common cause being infection from contaminated intravascular devices or following surgery (Miles, Denton and Kerr, 1998), the best precaution remains to avoid unnecessary interventions and ensure the sterility of all the tools used during these procedures.

1.2.6. *S. maltophilia* in the environment

In the environment, *S. maltophilia* is widely found in the rhizosphere, plants and soil although not as a phytopathogen but rather in beneficial associations eliciting favourable effects on its hosts such as favouring growth and health of plants (Ryan *et al.*, 2009). Its unique ability to survive under nutrient-limited conditions and its biochemical properties reveal it as a promising biotechnological tool for bioremediation and phytoremediation (Binks, Nicklin and Bruce, 1995; Mukherjee and Roy, 2016).

However, further studies are needed to assess the potential risk of spreading resistance genes from environmental sources to human pathogens since evidence suggests that environmental isolates carry an even greater amount of resistance genes than clinical isolates (Brooke, 2012). Hence, such application would require very controlled settings and careful monitoring.

1.2.7. *S. maltophilia* as a human pathogen

On the other hand, the clinical isolates of *S. maltophilia* are characterised by a higher rate of mutation and a great ability for adaptation to their host microbial environment. In fact, in their genomic functional analysis Crossman *et al.* (2008) identified a subgroup referred to as 'phylogenetic group A' of *S. maltophilia* that were very similar despite their unrelated sites of isolation and seemed to have a greater capacity to cause infection than other *S. maltophilia* strains (Crossman *et al.*, 2008). They shared resistance-nodulation-division (RND)-type efflux pumps genes conferring them additional antimicrobial resistance to a variety of drugs. Hence, the biggest fear is that, despite not being a very virulent organism, *S. maltophilia* could act as a reservoir of resistance for more commonly found pathogens such as *Pseudomonas aeruginosa*, *Serratia marcescens* and other pathogens that can be co-cultured with it (Crossman *et al.*, 2008).

1.2.8. Treatment of *S. maltophilia* infection

Treatment of *S. maltophilia* is particularly difficult due to its wide multidrug resistance; the current optimal treatment for *S. maltophilia* infection is trimethoprim/sulphamethoxazole (TMP-SMX) regime (Senol, 2004). However, with the spread use of antibiotics that favours selection of mutations leading to improved resistance, TMP-SMX resistant strains are becoming increasingly common (Brooke, 2012). A study suggests the acquisition of such resistance from plasmids and transposons containing *sul* genes (Toleman *et al.*, 2007). To overcome such resistance, combinational antibiotic regimes such as ticarcillin-clavulanic acid (TC-CL), a beta-lactam and TMP-SMX can be prescribed to treat highly resistant strains (Milne and Gould, 2012).

Other antibiotics prescribed to patients with allergies or resistant to the previously mentioned ones include tetracyclines such as minocycline, levofloxacin and doxycycline (Flamm *et al.*, 2016). However, it is important to highlight the lack of evidence of effectiveness of such antibiotic treatments *in vivo* and the need for controlled and disease-specific trials (Amin and Waters, 2014).

1.2.9. *S. maltophilia* in cystic fibrosis

S. maltophilia has been extensively studied in cystic fibrosis (CF) patients since it represents one of the major potential pathogens, being found in up to 30% of patients presenting bacterial infections (Denton *et al.*, 2000).

CF is an autosomal recessive genetic disease affecting multiple organs and is caused by a mutation on chromosome 7. The most common one consists of the deletion of a single amino acid, $\Delta F508$ (O'Sullivan and Freedman, 2009). Any

mutation on chromosome 7 results in the disruption of the cystic fibrosis transmembrane regulator (CFTR) protein which is a chloride channel found quite ubiquitously in the body on the membrane of epithelial cells and, importantly, plays a key role in the regulation and transport of salt and water (Bilton, 2008). Due to its ubiquitous location, malfunction of the CFTR leads to a plethora of disorders in every organ with a secretory function. In the pancreas, it can lead to malabsorption; some patients also exhibit biliary duct plugging and in the reproductive system, it can cause infertility. However, the main cause of death among CF patients is respiratory failure due to the lung damage produced by impairment of the mucociliary clearance apparatus and subsequent mucus plugging (Bilton, 2008). These lead to recurrent infections with *Staphylococcus aureus*, *P. aeruginosa*, *Haemophilus influenzae*, *Burkholderia cepacia* complex, *S. maltophilia* and *Achromobacter xylosoxidans*. Cystic fibrosis represents the most common inherited disease among the Caucasian population since about 1 in 25 Caucasians are believed to be carriers (Denton *et al.*, 2000). Treatment and management of the disease such as physiotherapy, DNase, antimicrobial therapy and hypertonic saline treatment have considerably extended the life expectancy of CF patients in the last decades, with most of them reaching adulthood and being able to carry a relatively normal life, attending education and contributing to the workforce (Bilton, 2008).

Effects on cystic fibrosis

Despite the high incidence of *S. maltophilia* in CF the question of whether it is a *true* pathogen contributing to deterioration of the lung function in patients is still controversial (Colin and Rabin, 2011). An initial study on the serological response in chronically infected CF patients showed increased antibody levels to *S. maltophilia* compared to patients only presenting acute infection (Waters *et al.*, 2011). It was thus proposed that chronic infection due to *S. maltophilia* could be considered a risk factor for exacerbation. Nevertheless, these findings are not enough to infer its role in deterioration of the lung function; further studies are needed to determine this causality by looking at the chronicity of the events (Colin and Rabin, 2011). In fact, the opposite could also be true; the presence of *S. maltophilia* could be a consequence of the progression of the disease without itself being responsible for the deterioration of lung function. In such cases, the bacterial infection would be more of a “secondary effect” to the progression of the disease rather than its driver. In addition, the subsequent evidence of rising titres of antibodies against *S. maltophilia* would thus be solely due to the presence of the bacteria in an already damaged lung (Colin and Rabin, 2011).

1.2.10. *S. maltophilia* in bronchiectasis

So far, *S. maltophilia* has not been studied in bronchiectasis as it is not as common as in CF (Boyton, 2012). Nevertheless, both diseases share the phenotype of accumulation of sputum in the lungs, which is a favourable reservoir for growth of bacteria and a main contributor to the high incidence of bacterial infections in patients affected by such diseases. Therefore, it is important to establish the differences and similarities of both diseases and of the impact of *S. maltophilia* infection in both. A lot can be learnt from previous studies in CF and similarly from the gaps in the understanding of *S. maltophilia* in CF such as the paradigm for the causal or consequential relationship between its chronic infection and disease progression.

2. Aims

The aims of this project were to:

1. Investigate the prevalence of *S. maltophilia* in bronchiectasis and its clinical impact;
2. Compare strains isolated from patients with bronchiectasis and patients with cystic fibrosis as well as environmental strains isolated from salad samples and determine whether the clinical strains and environmental ones differ;
3. Look at the inflammatory response to *S. maltophilia* assessing its pathogenicity.

3. Materials and Methods

3.1. Data collection

All the data from databases were extracted from TrakCare®, the NHS database: pulmonary function, BMI and other variables required for the calculation of patients' BSI were found in clinic notes and the serological parameters under the *blood results* section, the sputum bacteriology results from the clinical labs, under the *microbiology* section.

3.2. Isolation of *S. maltophilia* strains

Clinical *S. maltophilia* strains from bronchiectasis patients were isolated from sputum. When isolated from fresh sputum the following routine procedure was used: the sputum was weighed and an equal volume of Sputolysin (Calbiochem, Millipore (UK) Ltd., Watford) was added; then 1 in 10 dilutions in sterile saline were made and 10^{-2} and 10^{-4} dilutions were plated onto PIA –*Pseudomonas* Isolation Agar (Difco), BA –Blood Agar (Columbia agar base plus 5% horse blood –Oxoid) and CBA –Chocolate Blood Agar (Columbia agar base plus 5% horse blood, heated plus bacitracin, 0.35mg/ml). They were incubated at 37°C for 48h and colonies were subsequently counted (annexe 1).

Suspected *S. maltophilia* strains from PIA plates were then sub-cultured onto Nutrient Agar (NA) and MacConkey Agar plates for 24h at 37°C. In most cases, MacConkey agars should turn orange and colonies from the NA were picked to perform API® 20NE tests (BioMérieux), following the manufacturer's protocol. The results from the API® 20NE were recorded after both 24h and 48h incubation at 30°C and identity was subsequently confirmed entering the results in the apiweb™ online tool. Strains were stored in triplicate by taking out three single colonies into three different cryotubes containing 1ml of 10% skimmed milk (Oxoid) and stored at -70°C.

Some of the clinical samples were thawed from frozen neat sputum from previous studies stored at -70°C. Those were streaked straight onto PIA and BA using a loop. They were then incubated for 48h at 37°C and sub-cultured on NA and MacConkey agar. Subsequently, API® 20NE was performed as previously mentioned.

Isolation of environmental isolates of *S. maltophilia* from salad packs were homogenized with saline and plated onto selective VIA (Vancomycin Imipenem and Amphotericin B) medium for *S. maltophilia* –in addition to the PIA, BA and CBA plates. VIA was prepared following Denton's paper (Denton *et al.*, 2000) using 1mg of vancomycin (900µg/mg), 1mg of amphotericin B (80%) and 7mg of imipenem into

200ml of distilled water mixed with mannitol solution (Mast Diagnostics Ltd.) 100µl of the saline solution was spread onto the VIA plates and incubated at 37°C for 48h.

3.3. Antimicrobial Susceptibility Testing (AST)

3.3.1. Disk diffusion method

Strains were plated onto NA and incubated at 37°C for 24h and colonies were subsequently grown in nutrient broth (NB) enriched with 0.5% Yeast Extract (YE) (Oxoid) and incubated overnight at 37°C in an orbital incubator (Sanyo). 10 µl of the broth was diluted 1:100 in 0.85 % saline and used to flood seed DST agar plates. Excess solution was removed and discarded and the specific antibiotic discs were distributed on the agar plates which were then incubated overnight at 37°C. After incubation, sensitivity was recorded by measuring the diameter of the zone of inhibition around the disks and compared to the standard values given for each antibiotic disk by the manufacturer.

3.3.2. Etest method

S. maltophilia colonies grown on nutrient agar at 37°C for 24h were diluted into 10 ml of saline and cell density was adjusted by measuring the turbidity of the samples against a McFarland Standard 0.5 from Pro-Lab Diagnostics in a Nephelometer Sensititre® from Thermo Scientific. Diagnostic sensitivity testing (DST) agar plates were flood seeded with the bacterial suspension and excess solution was removed. Etest® strips from BioMérieux of the corresponding antibiotic were then placed at the centre of the plate using sterilised forceps. The plates were incubated at 37°C overnight and the minimum inhibitory concentration (MIC) was then recorded as the concentration which demonstrated inhibition of growth on the strip (annexe 2).

3.4. Pulsed-Field Gel Electrophoresis

3.4.1. Plug Preparation

Fresh *S. maltophilia* bacterial cells grown for 24h at 37°C on non-selective NA plates were suspended in 1ml SE buffer, in Eppendorf tubes and centrifuged for 2 mins at 13 000 rpm. The pellets were re-suspended in 500 µl of SE buffer but the volume was adjusted depending on the size of the pellet to give an uniform cell density for each isolate. The bacterial cells were encased in an agarose matrix made up of 1.5% low melt agarose (Bio-Rad Laboratories) in SE buffer (0.15g of low melt agarose in 10ml of SE buffer), previously microwaved for 15 secs and then 4 times 5 secs, gently mixing between every heating. An equal volume of 1.5% low melt agarose solution was added to the bacterial suspension in SE buffer and subsequently pipetted into the plug mold. Plugs were left at 4°C for 15 mins to

solidify. Plugs were then transferred into small bijoux containing 2 ml of lysis buffer and 10 μ l Triton X-100 (Sigma) and incubated overnight in a waterbath at 55°C. Plugs were then washed by removing the lysis buffer and replacing it with 2 ml of TE buffer for 30 mins at 4°C and repeating this step carefully another two times to ensure removal of any potential cellular debris that would prevent proper digestion in later steps.

3.4.2. Restriction Enzyme Digestion

A small portion of each plug of approximately 2 mm was cut and placed in an Eppendorf tube filled with 90 μ l of distilled water (DW) and 10 μ l of reaction buffer (10X REact® 2 buffer; Invitrogen™) and placed in a fridge at 4°C for 30 mins. The buffer was carefully removed using a fine Pasteur pipette and replaced by a solution containing 90 μ l of DW, 10 μ l of reaction buffer, 2 μ l of bovine serum albumin (BSA 1 mg/ml; New England BioLabs® Inc.), 2 μ l of dithiothreitol (DTT 1.5 mg/100 μ l DW) and 2 μ l of restriction enzyme (*Xba*I; Invitrogen™) in which the plugs were incubated overnight at 37°C in a waterbath.

3.4.3. Loading of Plug Slices and Running of the Gel

2L of 0.5% TBE buffer (Severn Biotech Ltd.) was made and 150 ml of it was used for making the gel, to which 1.5 g of pulsed-field agarose (Bio-Rad Laboratories) was added and the mix was microwaved for 1 min, then mixed and again microwaved for 1 min 10 sec. Once cooled, the agarose was poured into the gel mold previously cleaned with ethanol, and allowed to solidify for 30 mins. Finally, the plugs were placed in the wells and the gel was placed in the CHEF-DRII gel tank (Bio-Rad Laboratories) and the remainder of the 2L of TBE buffer was added. The chiller was set at 14°C and parameters were set as follows: initial time 2.9 secs, final time 35.4 secs, voltage 6 volts/cm, run time 20h.

3.4.4. Image Analysis

The gel was stained with gel red (7.5 μ l in 250ml of 0.1M NaCl) for visualization under UV trans-illumination using the Molecular Imager® GelDoc™ XR+ system with Image Lab™ software (Bio-Rad Laboratories). The exposure time and saturation were adjusted to obtain optimal pictures with distinct bands.

3.5. Virulence factors

3.5.1. Lipase

Specific agar for this test was composed of 85ml nutrient agar and 15ml of egg-yolk emulsion (Oxoid). The NA was autoclaved and the egg-yolk emulsion added after cooling to room temperature. A few colonies of each test isolate, taken from a NA plate, were inoculated into 10ml of NB containing 0.5% YE and incubated overnight at 37°C in an orbital incubator. The plates were then inoculated with the different isolates of *S. maltophilia* using a multiporin inoculator and incubated overnight at 37°C. Zones of opalescence indicated the production of lecithinase and an iridescent layer indicated lipolysis, which are markers of virulence. The positive control was an isolate of *P. aeruginosa* (ATCC).

3.5.2. Protease

Specific agar for this test was 100ml of brain heart infusion (BHI) broth (Oxoid) + 3% Bacteriological agar (Oxoid) and equal volume of 3% skimmed milk (100ml) (Oxoid). Each component was autoclaved separately and then mixed and poured into petri dishes. The same broth (NB+0.5% YE) culture for each test isolate, as used for the Lipase experiment was used to inoculate the Protease plates, using the multiporin inoculator and the plates were incubated at 37°C overnight. A clear zone around the bacterial growth indicated protease activity, which is a marker for virulence.

3.5.3. Elastase

Specific agar used to test elastase activity of the isolates was made of 100ml of autoclaved NA to which 0.3% of elastin Congo red (Sigma) was added before pouring the plates. The plates were then inoculated as described above overnight at 37°C.

3.6. Inflammatory markers in sputum

3.6.1. Preparation of spun sputum supernatant for MPO and NE assays

Sputum was collected in a sterile container and processed within 4h. Approximately 1ml was required for routine qualitative microbiology by the hospital laboratories. If there was enough left over, another 1ml was used for further quantitative and qualitative microbiology by the research laboratory and the remaining –if any– was then spun down at 23 200 rpm for 1h30mins at 4°C in the ultracentrifuge (Discovery S-100, Sorvall). The supernatant was then collected and frozen directly at -70°C for further studies, including the inflammatory markers assays detailed below (3.6.2. and 3.6.3.).

3.6.2. Myeloperoxidase (MPO) assay

MPO activity was measured by a chromogenic substrate assay. All reagents were preliminarily brought to room temperature. Myeloperoxidase from Calbiochem® and spun sputum samples were diluted as required in phosphate buffer solution by adding 1.5 µl of sample into 73.5 µl of buffer. 25 µl of standards or samples solution were added to the wells of a flat-bottomed 96 well microtitre plate (Costar®), each in duplicate. After that, 25 µl of tetramethylbenzidine (Sigma-Aldrich®) was added to each well and the plate was left at room temperature for 5 mins. The reaction was then halted by the addition of 50 µl of sulphuric acid (H₂SO₄) to each well and the absorbance was recorded at a dual wavelength of 450 and 50 nm and the MPO concentration was extrapolated from the standard curve and expressed as µg/ml. Since samples were all performed in duplicate, the mean of both values was calculated.

3.6.3. Free Elastase Activity (Neutrophil Elastase, NE) assay

Free elastase activity was measured by spectrophotometry using the synthetic substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val p-Nitroanilide (Sigma-Aldrich®). Standards (NE from Sigma-Aldrich®) and spun sputum samples (2 µl) were diluted as required in buffer (98 µl). 40 µl of the standard or sample solutions were added to each well of a flat-bottomed 96 well microtitre plate (Costar®). 200 µl of MeOSAAVpNa was added to 4.8 ml of buffer. 40 µl of this solution was added to each well and samples read immediately at 37°C for a minimum of 30 mins with readings every 2 mins; at an optical density (OD) of 405 nm. The rate of change in optical density was converted into elastase activity and expressed in µg/ml. The elastase concentration for each sample was determined in duplicate and the mean was calculated for each.

3.7. Data analysis

All the data were tested for normality using the Shapiro-Wilk test and according to the data distribution, either Kruskal-Wallis or Mann-Whitney U-test was used to compare groups. Chi squared (χ^2) was used to compare groups for categorical data. Data are presented as n (%), mean (standard deviation) or median [interquartile range], unless otherwise stated. Differences were considered significant when p-values were lower than 0.05. Analyses and graphs were made using the following softwares: SPSS Version 19 for Windows (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA, USA), as well as Microsoft Excel 2013.

4. Results

4.1. Prevalence of *S. maltophilia* in bronchiectasis

To have an idea of the importance of *S. maltophilia* in our cohort of bronchiectasis patients, a retrospective analysis of the database was performed by looking at the microbiology of all the bronchiectasis patients in a 3-years' timeframe (2013-2016) using TrakCare®, the NHS database. Out of 458 patient found in clinic notes from the last 3 years, a total of 37 patients (~8%) grew *S. maltophilia* at some point.

The first observation is that most of the patients growing *S. maltophilia* in their sputum belong to the higher spectra of bronchiectasis severity. Most of them have severe bronchiectasis and the rest are moderate, with a single patient described as having mild bronchiectasis (Fig. 4.1).

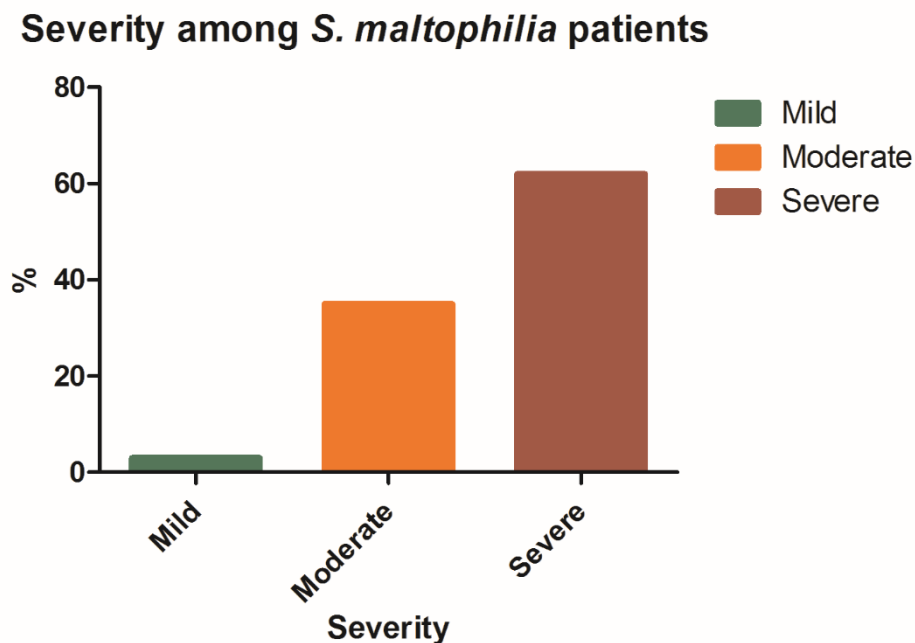


Figure 4.1 Severity of bronchiectasis among the patients who grew *S. maltophilia* from 2013 to 2016. The majority were severe (62%) and moderate (35%) whereas only one (3%) had mild bronchiectasis.

All the episodes of *S. maltophilia* in these 37 patients' sputum reported in their microbiology notes were collected using TrakCare® and accounted for a total of 242. In the majority of these cases (186 episodes, 76.9%), *S. maltophilia* was isolated on its own, as a monoculture whereas in the remaining 85 episodes, it was found along with other pathogen(s). The most common co-cultured organisms were *P. aeruginosa*, *S.aureus* and fungi (Fig. 4.2.).

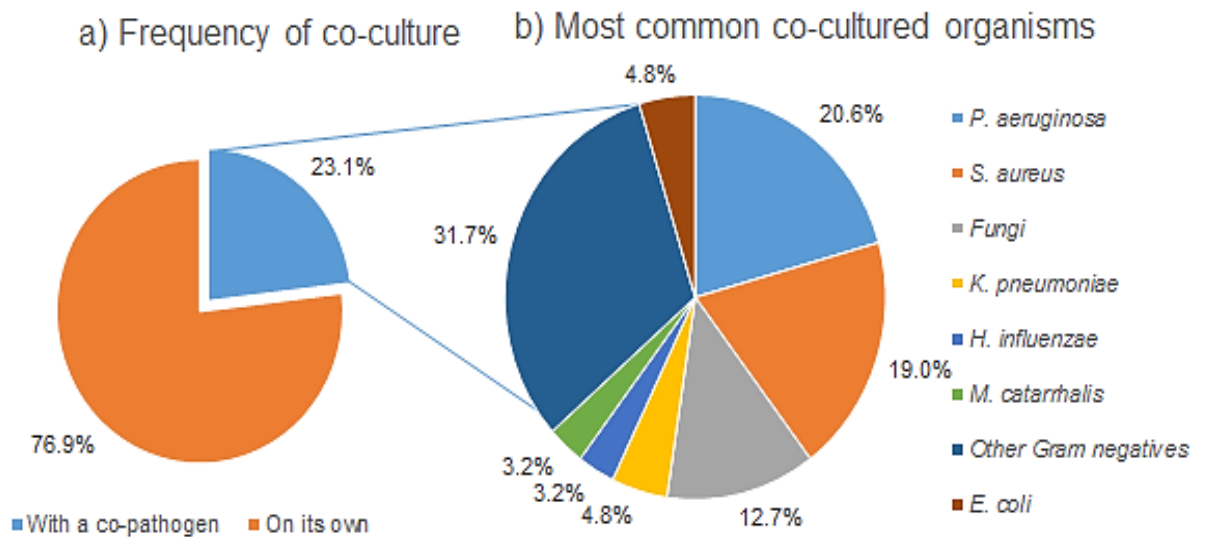


Figure 4.2 Frequency and identity of co-pathogens isolated along with *S. maltophilia*. a) In 76,9% of the cases, *S. maltophilia* was isolated on its own. b) When it was with other organism(s) the most common ones were *P. aeruginosa* (20,6%), *S. aureus* (19,0%) and fungi (12,7%).

Out of the 37 patients from which *S. maltophilia* was ever reported in the sputum, 20 had a single isolation only and the remaining 17 presented two or more episodes within a year, at least three months apart –hence, considered as *chronically infected*. Interestingly, there was no significant difference (p-value = 0.62) in the BSI score between the two groups (i.e. chronically infected and single isolation) (Fig. 4.3).

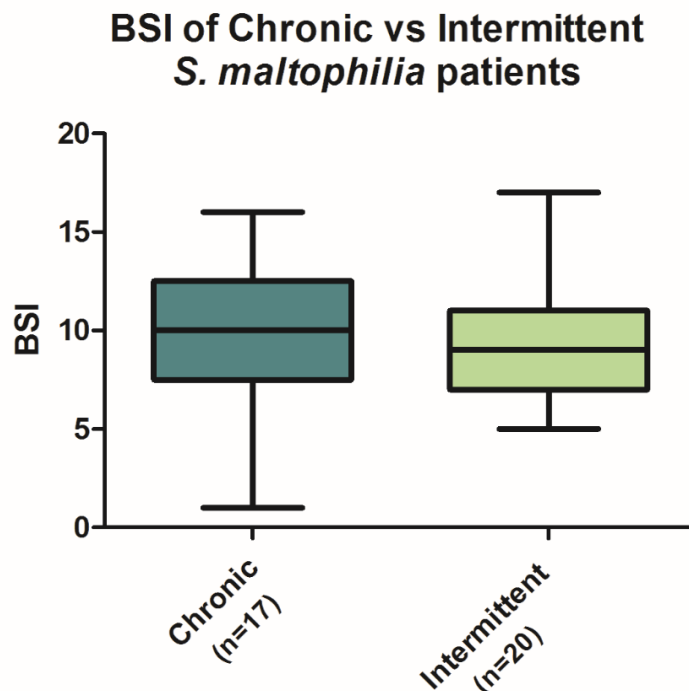


Figure 4.3 Comparison of BSI score of patients from which *S. maltophilia* was isolated chronically or intermittently in sputum samples. No significant difference of BSI was found between the two groups (p-value = 0.62).

The initial plan was to compare white cell counts, ESR and CRP between single versus chronically infected groups but this was abandoned as there was a lot of missing data.

Instead, another more exhaustive database solely containing data from episodes of bronchiectasis patients during IV antibiotic treatment was studied. Interestingly, *S. maltophilia* had a proportionally higher incidence but not reaching conventional statistical significance (~13%, $p = 0.13$) when compared to its incidence among entire bronchiectasis cohort (Fig. 4.4).

Incidence of *S. maltophilia* in the entire bronchiectasis cohort vs bronchiectasis patients on IV

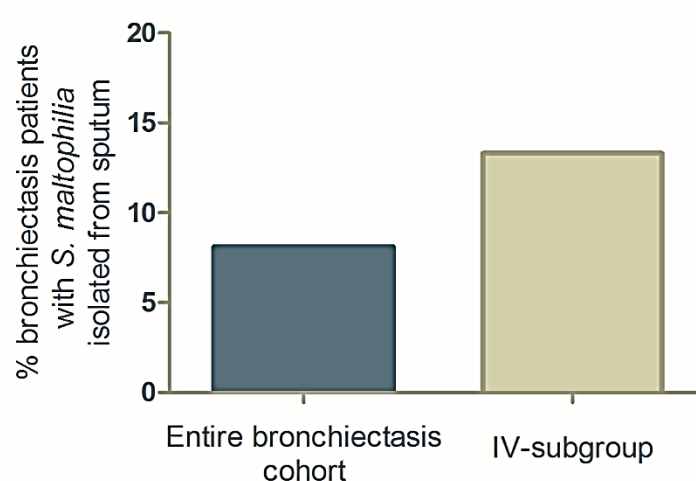


Figure 4.4 Comparison the incidence of *S. maltophilia* among the entire bronchiectasis cohort and the cohort of bronchiectasis patients on IV antibiotics.

This database was divided into three groups: (1) 11 episodes of *S. maltophilia* isolations at baseline (before IV treatment) with complete data available were identified and they were then matched in a 1:2:2 with episodes of (2) *P. aeruginosa* and (3) *H. influenzae* from patients of same gender and similar age and BSI –when possible (annexe 3). Such ratio was chosen due the low incidence of *S. maltophilia* episodes encountered and they were compared to *P. aeruginosa* and *H. influenzae* since these are the most common pathogens in bronchiectasis (Dimakou *et al.*, 2016).

General variables about the lung function such as FEV₁ % predicted and FVC % predicted were compared as well as serological markers including white cells counts (WCC), platelets levels, ESR and CRP.

As shown below in Table 4.1, there was no statistically significant difference in spirometry, white cell count, ESR or CRP between groups at the start of an exacerbation: p-value is always equal to or greater than 0.05 ($p \geq 0.05$).

Table 4.1 Lung function and serological variables for each group of episodes (*S. maltophilia*, *P.aeruginosa* and *H. influenzae*) at baseline, before IV treatment.

| | <i>S. maltophilia</i> (n=11) | <i>P. aeruginosa</i> (n=22) | <i>H. influenzae</i> (n=22) | p-value |
|--|---------------------------------|--------------------------------|--------------------------------|---------|
| Lung function: | | | | |
| – FEV1 % predicted | 36.3 [30.7 – 59.0] | 51.9 [41.6 – 60.5] | 52.0 [30.7 – 78.0] | 0.25 |
| – FVC % predicted | 65.3 [54.3 – 83.8] | 72.7 [67.2 – 79.8] | 70.0 [52.8 – 105.6] | 0.45 |
| WCC [4.0-11.0 x 10 ⁹ /l] | 6.8 [5.9 – 9.4] | 9.3 [3.6 – 12.9] | 8.0 [6.7 – 9.8] | 0.05 |
| Platelets [150-400 x10 ⁹ /l] | 271 [245 – 331] | 326 [272 – 436] | 308 [217 – 406] | 0.45 |
| ESR [3-15 mm/hr] | 28 [16 – 77] | 27.5 [19 – 39.0] | 15.5 [5.7 – 32.7] | 0.06 |
| CRP [0-5 mg/l] | 12.0 [4.4 – 31.0] | 13.0 [9.0 – 33.7] | 9.0 [4.0 – 34.2] | 0.44 |

However, it is worth noting that the p-value is close to being significant for two of the variables: white cell counts (WCC) and ESR. When looking more closely at the data, the WCC ranges among normal values despite the different medians in each group.

On the other hand, regarding ESR, the *S. maltophilia* group shows a similar value to that of *P. aeruginosa*, which both fall above the normal range (3-15 mm/hr) whereas the median value for the *H. influenzae* group is a lot closer to the normal range (Table 4.1).

Table 4.2 Variables for each group of episodes (*S. maltophilia*, *P.aeruginosa* and *H. influenzae*) after completion of the 14-days IV antibiotic course.

| | <i>S. maltophilia</i> (n=11) | <i>P. aeruginosa</i> (n=22) | <i>H. influenzae</i> (n=22) | p-value |
|--|---------------------------------|--------------------------------|--------------------------------|--------------|
| Improvement in Lung function: | | | | |
| – FEV1 % predicted | 2.1 [-1.2 – 4.6] | 1.7 [0.0 – 4.8] | 1.3 [-2.4 – 4.4] | 0.86 |
| – FVC % predicted | 2.9 [-2.7 – 12.7] | 5.8 [-2.9 – 7.6] | 4.5 [-7.1 – 8.5] | 0.94 |
| WCC [4.0-11.0 x 10 ⁹ /l] | 6.6 [5.1 – 7.1] | 9.8 [7.6 – 11.8] | 7.6 [6.1 – 8.6] | 0.003 |
| Platelets [150-400 x10 ⁹ /l] | 284 [256 – 327] | 298 [249 – 403] | 280 [228 – 389] | 0.73 |
| ESR [3-15 mm/hr] | 19 [14.0 – 41.0] | 22 [17.0 – 27.5] | 9 [4.0 – 19.5] | 0.006 |
| CRP [0-5 mg/l] | 6.0 [2.0 – 25.0] | 9.5 [5.0 – 14.7] | 3.0 [2.0 – 10.2] | 0.23 |

Further longitudinal analysis looked at the same variables after the 14-day IV antibiotic treatment and found this time a significant difference (p-value < 0.05) between the three groups for both the WCC and ESR. The white cell count was slightly higher in the *P. aeruginosa* group compared with the other groups although the median value remained within the normal range (Table 4.2).

More interestingly, regarding the ESR levels, antibiotic treatment appeared to bring the values into the normal range among the group that had tested positive for *H. influenzae* at baseline. However, the values for both the *S. maltophilia* and *P. aeruginosa* remained above the normal range (Table 4.2). In fact, further statistical analysis revealed a statistical difference in the ESR values between the *S. maltophilia* group and the *H. influenzae* group (p-value = 0.013) and between the *P. aeruginosa* group and the *H. influenzae* group (p-value = 0.003) but not between the *S. maltophilia* and *P. aeruginosa* groups (p-value = 0.85).

4.1.1. Incidence of *S. maltophilia* among the IV sub-group

Following the relatively high incidence of *S. maltophilia* among the IV sub-group, its incidence in this cohort was further analysed.

All the recorded courses of IV antibiotics between 2013 and 2016 were collected and accounted for 237 14-day IV antibiotic courses from 74 different patients (some were under long-term preventative IV treatment; others were related to treatment for exacerbations according to the BTS guidelines). The organisms grown at baseline (day 0) (Fig. 4.5 (a)) were compared to those isolated from sputum at the end of the 14-day IV antibiotic course (Fig. 4.5 (b)).

Organisms prior to IV course - out of the 237 episodes

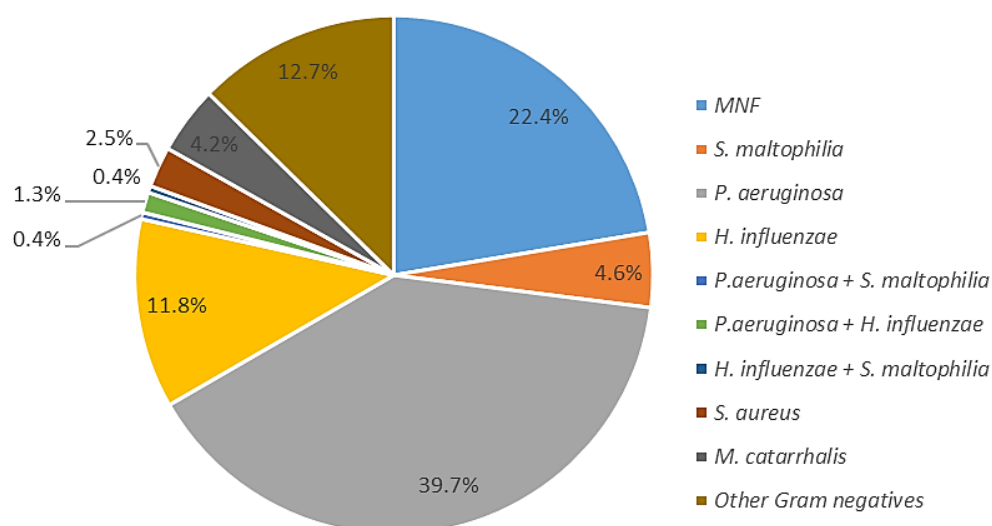


Figure 4.5 (a) Organisms isolated at baseline prior to 237 episodes of 14-day IV course (MNF = Mixed Normal Flora).

Organisms at end of IV course - out of 237 episodes

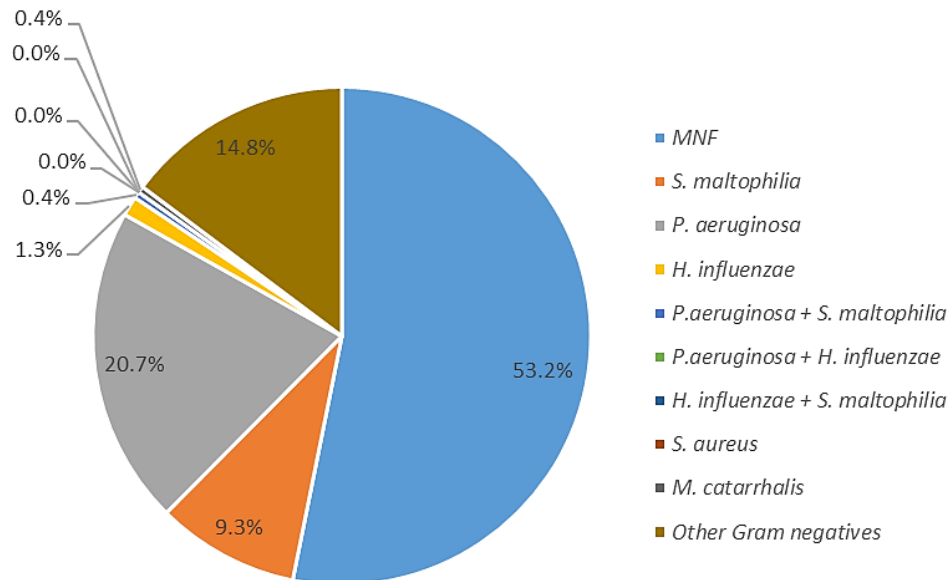


Figure 4.5 (b) Organisms isolated at the end of the 237 episodes of 14-day IV course.

The main observation regarding *S. maltophilia* is that its incidence increases after the IV antibiotic course as opposed to *P. aeruginosa* for which occurrence is almost halved by the IV antibiotic course (Fig. 4.5 (a) & (b)). However, these results could be disproportionally influenced by single individuals on regular IVs that account for a significant proportion of incidence of a particular bacterium. Therefore, to confirm the previous observations, the organisms were also compared per patient rather than IV antibiotic course, looking at the organisms isolated at baseline and end of 14-day IV antibiotic course of the earliest IV antibiotic course recorded since 2013 (Fig. 4.6 (a) & (b)).

Organisms prior to IV course - out of the 74 patients

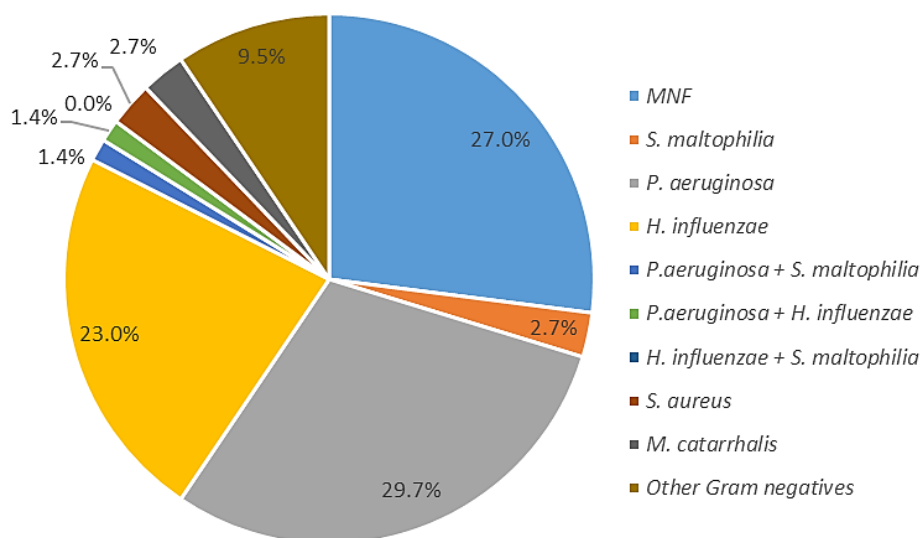


Figure 4.6 (a) Organisms isolated at baseline prior to the first IV-course starting from 2013 recorded for each of the 74 patients from the IV database.

Organisms at the end of IV course - out of the 74 patients

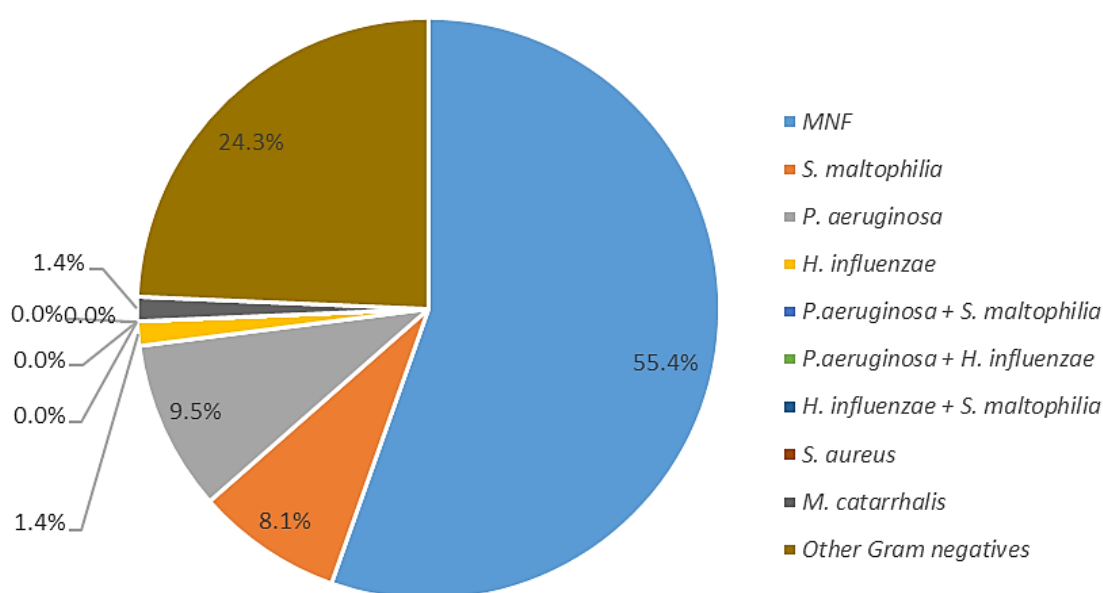


Figure 4.6 (b) Organisms isolated at the end of the first IV-course starting from 2013 recorded for each of the 74 patients from the IV database.

Interestingly, a similar trend was observed, consistent with the previous comparison of organisms per episodes (Fig. 4.5 (a) & (b)): *H. influenzae*, *S.aureus* and *P. aeruginosa* were all relatively successfully eliminated following the 14-day IV antibiotic course whereas *S. maltophilia* appeared at a higher incidence after the IV-course (Fig. 4.6 (a) & (b)).

To check whether IV treatment had triggered the appearance of *S. maltophilia* among the 10 patients from the IV database that were found to isolate it in their sputum, their full microbiology was examined on TrakCare®. All recorded episodes of *S. maltophilia* were summarised and their chronology with respect to the start of IV therapy was analysed (Fig. 4.7).

For most of them (80%), IV-treatment appeared to trigger an increase in incidence of *S. maltophilia*: for four patients, IV treatment appeared to switch the frequency of isolation of *S. maltophilia* from intermittent to chronic, for three patients, it went from non-existent to chronic and in one patient *S. maltophilia* was isolated intermittently following IV-treatment (Fig. 4.7).

History of the frequency of isolation of *S. maltophilia* among the 10 patients with *S. maltophilia* from the IV sub-group

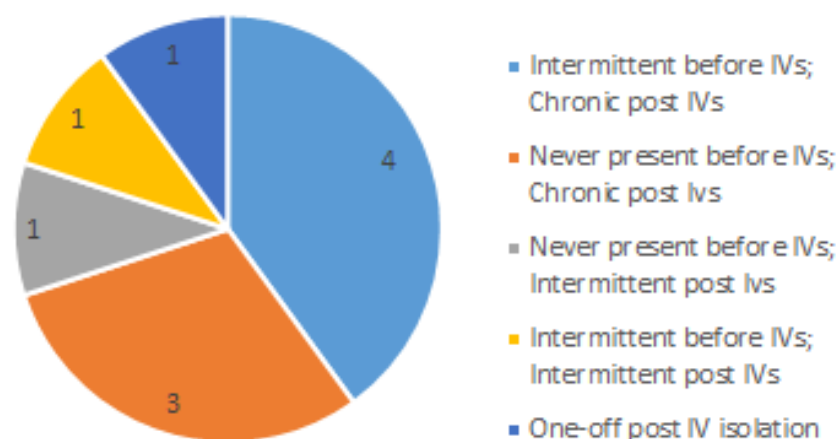


Figure 4.7 History of the frequency of isolation of *S. maltophilia* among the 10 patients with *S. maltophilia* from the IV sub-group.

To see whether this was due to a particular antibiotic that favoured *S. maltophilia*, a closer look was given to the specific antibiotic given to each of the 10 patients (Figure 4.8).

Type of IV antibiotic given to the 10 *S. maltophilia* patients

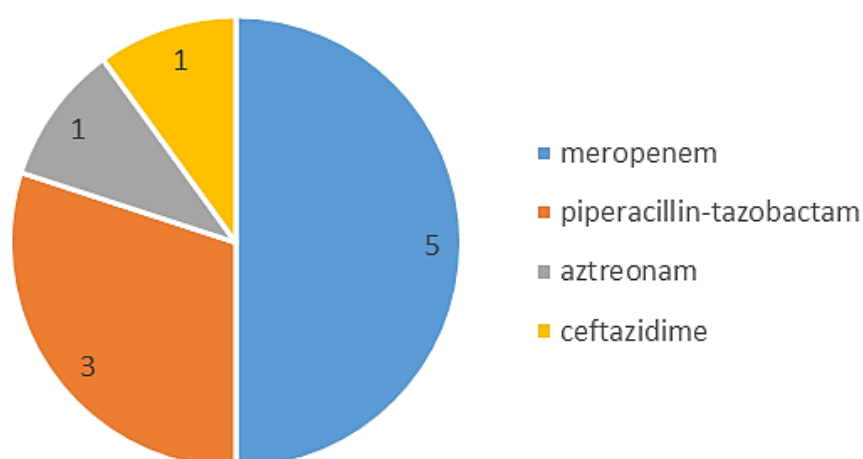


Figure 4.8 Type of IV antibiotic given to the 10 *S. maltophilia* patients from the IV subgroup.

As shown in Figure 4.8, the 10 patients were given a variety of antibiotics and thus the subsequent isolation of *S. maltophilia* in their sputum could not be attributed to a particular antibiotic but perhaps to IV-treatment in general.

4.1.2. Incidence of *S. maltophilia* following nebulised versus IV antibiotic treatment

Following the interesting observations made on the IV sub-group, another retrospective data analysis was performed looking at the microbiology of patients currently on long-term IV (every 2-3 months) and another set of patients currently on long-term nebulised antibiotics –it is worth noting that there was some overlap between the two sub-groups with some patients receiving both long term IV and nebulised antibiotic treatment, these were counted as part of the IV sub-group. The list of current patients in each category comprised 16 individuals. Again, their microbiology was examined using TrakCare® and noting any episode of *S. maltophilia* prior or post to long-term antibiotic therapy. Then, a longitudinal comparison between their most common colonising organism prior to the long-term antibiotic treatment and 1-year follow-up was performed (Fig. 4.9 (a), (b), (c) & (d)).

Organisms prior to long-term nebulised antibiotic therapy (n=16)

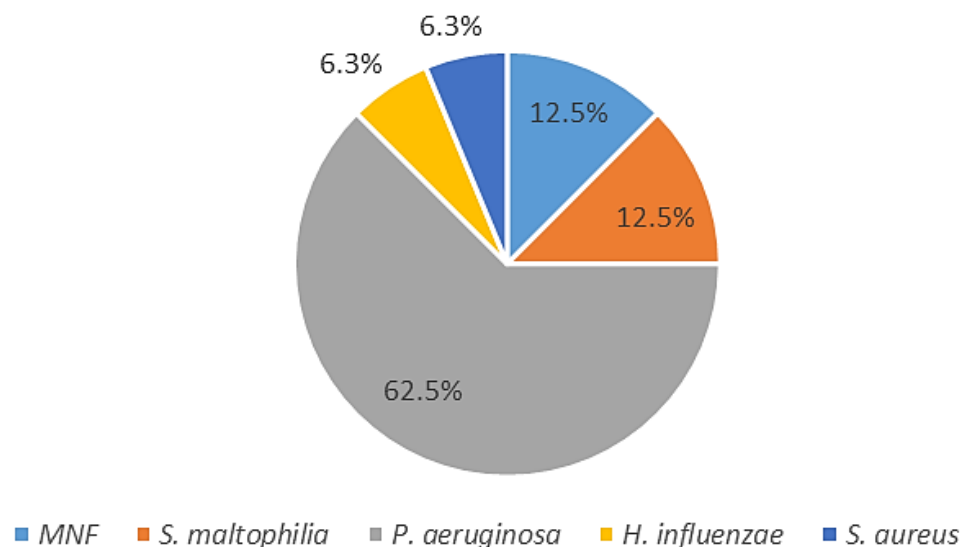


Figure 4.9 (a) Dominant organisms isolated prior to long-term nebulised antibiotic therapy.

Organisms 1-year after long-term nebulised antibiotic therapy (n=16)

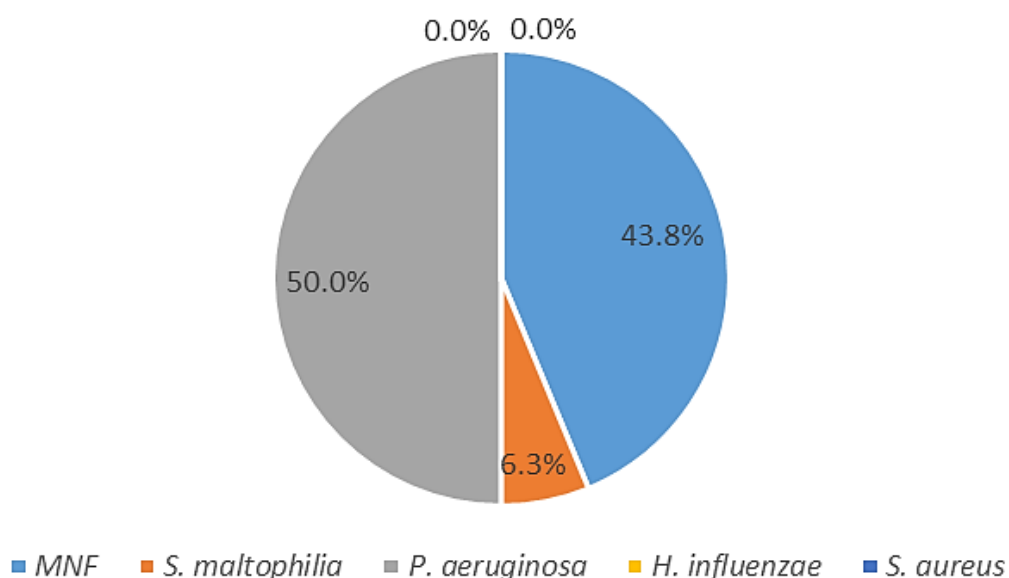


Figure 4.9 (b) Dominant organisms isolated 1 year after long-term nebulised antibiotic therapy.

Organisms prior to long-term IV antibiotic therapy (n=16)

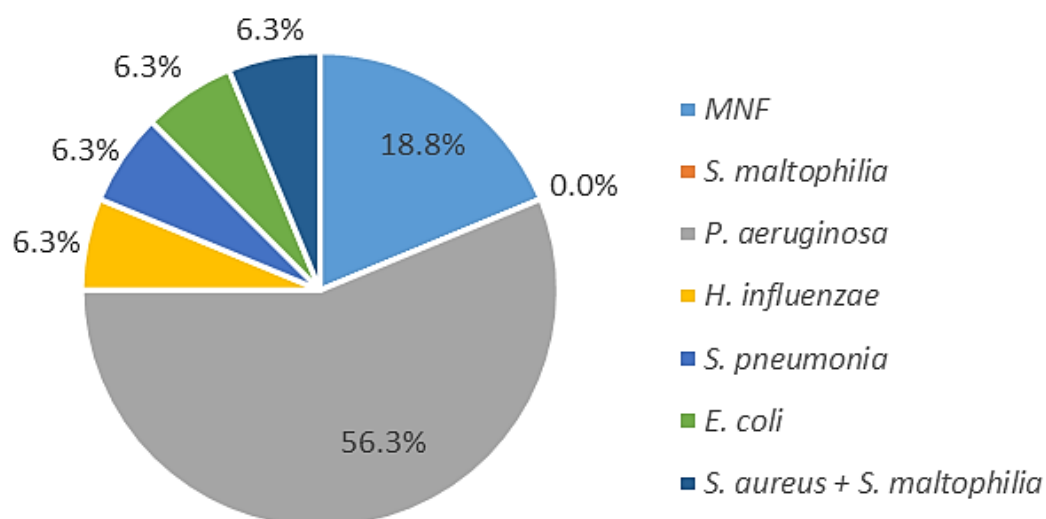


Figure 4.9 (c) Dominant organisms isolated prior to long-term IV antibiotic therapy.

Organisms 1-year after long-term IV antibiotic therapy (n=16)

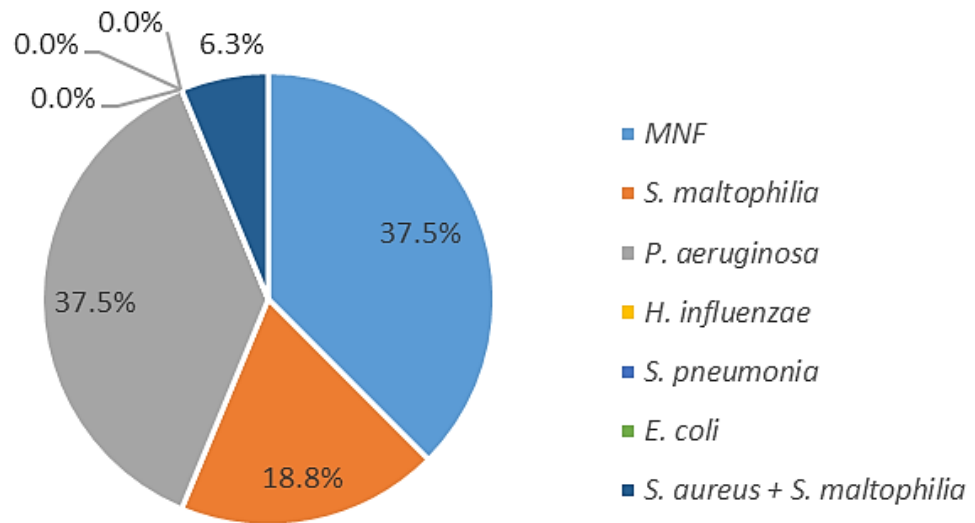


Figure 4.9 (d) Dominant organisms isolated 1 year after long-term IV antibiotic therapy.

Remarkably, the patterns were very different between the two groups: long-term nebulised antibiotic treatment did not seem to have any increasing effect on the incidence of *S. maltophilia* (Fig. 4.9 (a), (b), (c) & (d)). The history of the one patient where *S. maltophilia* persisted throughout the treatment was carefully examined and it was observed that they had become chronically colonised by *S. maltophilia* in July 2013 following a course of IV-meropenem in April 2013, prior to the start of their long-term nebulised antibiotic therapy started in November 2013.

On the other hand, a similar trend to the ones previously reported among IV patients was observed again among the smaller 16 patients' cohort currently undergoing preventative long-term IV antibiotic therapy, four of which are also on regular nebulised antibiotics. *S. maltophilia* seemed to be favoured (p -value < 0.001) by the IV regime and showed a higher incidence following one year of IV therapy compared to its incidence at baseline of treatment. (Fig. 4.9 (c) & (d)).

4.2. Comparison of bronchiectasis, CF and environmental strains

4.2.1. Gathering of the strains

The first 6 months of the project included active tracking of patients attending clinics and processing of their sputum in search for fresh *S. maltophilia* specimens (using the isolation methods described in the materials and methods section with graphic illustrations of the characteristic appearance of *S. maltophilia* available in the annexe 1). However, the search revealed to be quite slow and unfruitful due to the low incidence of the bacteria and another approach was thus added: records were examined for potential *S. maltophilia* stored in frozen sputum samples from previous studies. These were thawed and processed like the fresh specimens.

Eventually, a library of 18 strains isolated from sputum of 12 different bronchiectasis patients (10 from fresh sputum, 8 from recovered frozen sputum) was gathered. Another 4 environmental strains were isolated from environmental samples (mixed salad leaves and spinach). In total, 22 isolates of *S. maltophilia* constituted the library of strains used for the wet lab experiments (annexe 4). Another 10 isolates of *S. maltophilia* strains obtained from sputum of CF patients were provided by Dr. Catherine Doherty from the extensive library of strains accumulated over in the CF Strain Repository.

4.2.2. Comparison of antimicrobial resistance

To compare strains from clinical and environmental origin, an antimicrobial resistance experiment was designed based on the suggestions from the literature that environmental strains usually show a greater resistance pattern than clinical strains (Youenou *et al.*, 2015).

First, the disc diffusion method was used to identify the antibiotics worth investigating. Strains were tested against imipenem (IMI10), meropenem (MEM10), tobramycin (TN10), doxycycline (DO30) and trimethoprim/sulfamethoxazole (TMP-SXT) (TS25).

Due to the lack of guidelines regarding antimicrobial testing susceptibility (AST) breakpoints for *S. maltophilia* from both the Clinical and Laboratory Standards Institute (CLSI) (Nicodemo *et al.*, 2004) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the only antimicrobial agent for which zone diameter breakpoints specific to *S. maltophilia* were available was TMP-SXT (TS25) (<http://www.eucast.org>. 2016); for imipenem, meropenem and tobramycin, values from *P. aeruginosa* were used, considering the initial classification of *S. maltophilia* as a *Pseudomonas* species.

Unfortunately, for doxycycline, no value was available for either bacterial species or any other related one so it was not possible to accurately predict resistance or susceptibility but diameter breakpoints were still recorded and can be found in table 4.3.

In addition to the disc diffusion method, antimicrobial susceptibility of bronchiectasis and environmental strains was also tested using the Etest method. Strips for the same antibiotics as for the disc diffusion method were used: imipenem (IP), meropenem (MP), tobramycin (TM), doxycycline (DO) and trimethoprim-sulfamethoxazole (TS) as well as minocycline (MC) –for which susceptibility results were frequently reported from the microbiology laboratories of the hospital for strains that exhibited resistance to TMP-SXT.

On the other hand, the 10 CF strains provided by Dr. Catherine Doherty were also tested for antimicrobial susceptibility to imipenem, meropenem, tobramycin, doxycycline and trimethoprim-sulfamethoxazole using the disc method (Table 4.4) to compare resistance patterns to that of strains isolated from bronchiectasis patient.

For the disc diffusion method, diameters are reported in the tables below and for the Etest, MIC values are reported. A colour code was used to distinguish results corresponding to resistance (red), intermediate resistance (orange) and sensitivity (green) to the respective antibiotic (Tables 4.3 and 4.4).

There was a good correlation of the results between the Etest and disc method: as expected, all strains were resistant to imipenem, which is a characteristic of *S. maltophilia*. However, there was some discrepancy regarding resistance to TMP-SXT: the disc method identified more strains as being resistant to it than the Etest method (Table 1). The general pattern of susceptibility revealed the highest sensitivity of strains isolated from patients with bronchiectasis was minocycline, tobramycin and TMP-SXT, respectively.

It is worth noting that environmental strains isolated from salad samples did not appear to have a greater resistance pattern than the clinical strains as expected from literature (Youenou *et al.*, 2015). However, this might be due to the limited sample size and further studies with a higher number of samples are needed to confirm this hypothesis.

Table 4.3 Antimicrobial Sensitivity Testing of *S. maltophilia* strains isolated from bronchiectasis patients and environmental samples

| Strain | Disc Diffusion Method | | | | | Etest | | | | | |
|---------------|-----------------------|---------------|--------------|--------------|--------------|---------------|---------------|---------------|---------------|---------------|---------------|
| | IMI10 (mm) | MEM10 (mm) | TN10 (mm) | DO30 (mm) | TS25 (mm) | IP (µg/ml) | MP (µg/ml) | TM (µg/ml) | DC (µg/ml) | TS (µg/ml) | MC (µg/ml) |
| Patient 1 (1) | 6* | 26 | 32 | 36 | 36 | 32 | 3 | 0.125 | 0.19 | 0.064 | 0.25 |
| Patient 1 (2) | 6* | 32 | 28 | 34 | 40 | 32 | 0.75 | 1 | 0.75 | 0.047 | 0.125 |
| Patient 2 (1) | 6* | 6* | 6* | 32 | 6* | 32 | 32 | 256 | 1.5 | 6 | 0.75 |
| Patient 2 (2) | 6* | 6* | 6* | 28 | 6* | 32 | 32 | 256 | 3 | 32 | 1 |
| Patient 3 (1) | 6* | 26 | 22 | 25 | 32 | 32 | 2 | 0.75 | 2 | 0.016 | 4 |
| Patient 3 (2) | 6* | 19 | 18 | 23 | 26 | 32 | 2 | 4 | 2 | 0.032 | 0.25 |
| Patient 4 (1) | 6* | 30 | 25 | 28 | 28 | 32 | 1 | 0.25 | 0.50 | 0.064 | 0.25 |
| Patient 4 (2) | 6* | 25 | 24 | 31 | 30 | 32 | 1 | 1 | 0.75 | 0.64 | 0.094 |
| Patient 5 (1) | 12 | 20 | 24 | 22 | 6* | 16 | 1 | 0.5 | 3 | 0.25 | 1 |
| Patient 5 (2) | 11 | 20 | 26 | 20 | 6* | 32 | 2 | 0.75 | 3 | 2 | 0.25 |
| Patient 5 (3) | 17 | 25 | 30 | 27 | 25 | 6 | 0.75 | 0.38 | 1.5 | 0.047 | 1.5 |
| Patient 6 | 6* | 18 | 20 | 23 | 30 | 32 | 6 | 1.5 | 1 | 0.047 | 0.064 |
| Patient 7 | 6* | 25 | 22 | 25 | 34 | 32 | 2 | 0.38 | 1 | 0.064 | 0.19 |
| Patient 8 | 6* | 29 | 26 | 29 | 32 | 32 | 2 | 0.75 | 1 | 0.032 | 0.125 |
| Patient 9 | 6* | 6* | 15 | 32 | 6* | 32 | 32 | 2 | 3 | 0.125 | 0.25 |
| Patient 10 | 6* | 25 | 23 | 28 | 30 | 32 | 3 | 1 | 0.75 | 0.047 | 0.125 |
| Patient 11 | 6* | 6* | 22 | 38 | 38 | 32 | 32 | 1.5 | 0.38 | 0.064 | 0.047 |
| Patient 12 | 6* | 21 | 6* | 20 | 29 | 32 | 2 | 0.75 | 3 | 2 | 0.25 |
| Salad 1 | 6* | 17 | 27 | 27 | 6* | 32 | 0.5 | 2 | 2 | 0.125 | 1 |
| Salad 2 | - | - | - | - | - | 4 | 0.5 | 8 | 1 | 0.19 | 0.5 |
| Salad 3 | 6* | 32 | 24 | 30 | 34 | 32 | 0.5 | 1 | 1.5 | 0.032 | 0.125 |
| Salad 4 | 6* | 21 | 18 | 24 | 31 | 32 | 0.38 | 16 | 4 | 0.25 | 0.125 |

*6 = diameter of the disc, organism is completely resistant in such case.

Diameter breakpoints (mm) for each antibiotic disc (from EUCAST 2011):

IMI10 (Imipenem): R < 18, I : 18 – 20 , S > 20**

MEM10 (Meropenem): R < 18, I : 18 – 24, S > 24**

TS25 (TMP-SXT): R < 16, S ≥ 16

TN10 (Tobramycin): R < 16, S ≥ 16**

MIC values (µg/mm) for Etest results interpretation (from CLSI 2015):

MC (Minocycline): R > 16, I : 16 – 4, S < 4

TS (TMP-SXT): R > 4, I : 4 – 2, S < 2

IP (Imipenem): R > 8, I : 8 – 2, S < 2**

MP (Meropenem): R > 8, I : 8 – 2, S < 2**

TM (Tobramycin): R > 16, I : 16 – 4, S < 4**

DO30 and DC (Doxycycline): no value was available for *S. maltophilia* or any related species

** values of *P. aeruginosa* used

– Resistant (R) – Intermediate (I) – Sensitive (S)

Table 4.4 Antimicrobial Sensitivity Testing of *S. maltophilia* strains isolated from CF patients

| # | IMI10 (mm) | MEM10 (mm) | TN10 (mm) | DO30 (mm) | TS25 (mm) |
|-------|---------------|---------------|--------------|--------------|--------------|
| C1932 | 6* | 6* | 6* | 22 | 21 |
| C1954 | 6* | 20 | 6* | 21 | 32 |
| C1972 | 6* | 14 | 20 | 17 | 15 |
| C1970 | 6* | 6* | 6* | 25 | 20 |
| C1953 | 6* | 22 | 19 | 22 | 20 |
| C1969 | 6* | 10 | 6* | 25 | 25 |
| C1914 | 6* | 34 | 35 | 35 | 33 |
| C1957 | 6* | 6* | 13 | 27 | 28 |
| C1950 | 6* | 6* | 30 | 27 | 30 |
| C1936 | 6* | 22 | 6* | 18 | 25 |

*6 = diameter of the disc, organism is completely resistant in such case.

Diameter breakpoints for each antibiotic disc (from EUCAST 2011):

IMI10 (Imipenem): R < 18, I : 18 – 20 , S > 20** MEM10 (Meropenem): R < 18, I : 18 – 24, S > 24**

TS25 (TMP-SXT): R < 16, S ≥ 16

TN10 (Tobramycin): R < 16, S ≥ 16**

DO30 (Doxycycline): no value was available for *S. maltophilia* or any related species

** values of *P. aeruginosa* used

– Resistant (R) – Intermediate (I) – Sensitive (S)

Additionally, there is a clear difference in resistance patterns between bronchiectasis and cystic fibrosis strains: isolates from cystic fibrosis patients exhibit a much wider resistance pattern than the bronchiectasis ones. Most strains showed resistance or intermediate resistance to imipenem, meropenem and tobramycin, making TMP-SXT the most efficient antibiotic.

Finally, it is interesting to highlight the change in resistance pattern of some strains isolated from same patients (e.g. patient 5(1) and (3) and patient 3(1) and (2), disc diffusion method).

To check whether this was due to a loss of resistance or simply a change of strain, pulsed-field gel electrophoresis was performed on the five sets of strains that belonged to same patients (Fig. 4.10).

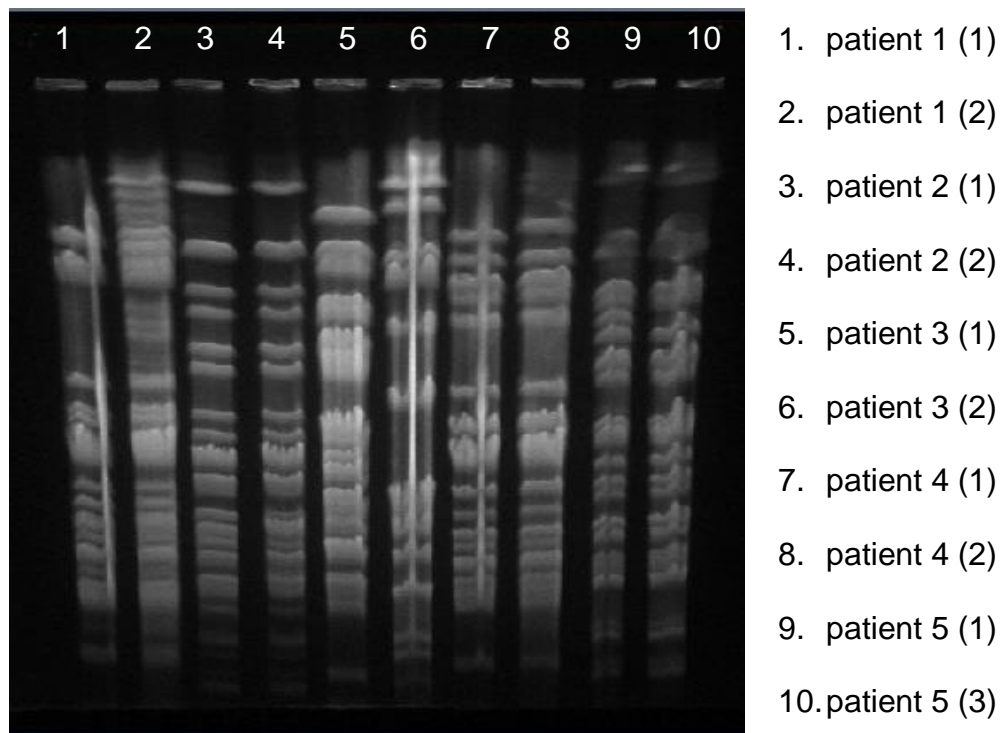


Figure 4.10 PFGE of strains isolated from bronchiectasis patients showing fingerprinting

Interestingly, the pulsed-field gel electrophoresis revealed that the strains from patient 1 and 3 had changed over time: they exhibited more than a 3-band difference, thus considered as being different strains whereas the others remained the same despite being isolated at different time points.

The difference in strains can explain the different resistance pattern of the isolates from patient 3. However, the putative loss of resistance observed by disc diffusion in the isolates from patient 5 is not due to a change in the strain (further evidence supporting this is the fact that the three isolates from patient 5 had been isolated in a 14-day period during a meropenem antibiotic course –annexe 4). A plausible explanation for the loss of resistance is that resistance genes come at a fitness cost and in scenarios where they are not essential, they could potentially be selectively lost (Andersson and Hughes, 2010).

Moreover, another specificity of the set of strains from patient 3 is that it is the only one from sputum samples collected at different exacerbation times; all the others were collected in close proximity during trials prior and after antibiotic treatments, generally in a 14-day period at most. In contrast, the set of strains from patient 3 had been collected at very different time points: one was rescued from frozen sputum from a past study whereas the other one was collected at a clinic visit during the time of this project.

4.2.3. Comparison of virulence factors

For further comparison between environmental and clinical strains, some virulence experiments were performed that looked at lipase, elastase and protease activity, all common virulence markers found in bacteria (Table 4.5).

Table 4.5 Virulence factors activity for each *S. maltophilia* strain

| STRAIN REF | LIPASE | PROTEASE | ELASTASE |
|---------------|--------|----------|----------|
| PATIENT 1 (1) | +++ | ++ | - |
| PATIENT 1 (2) | - | +/- | - |
| PATIENT 2 (1) | ++ | + | - |
| PATIENT 2 (2) | - | +/- | - |
| PATIENT 3 (1) | ++ | +++ | - |
| PATIENT 3 (2) | + | +++ | + |
| PATIENT 4 (1) | ++ | ++ | - |
| PATIENT 4 (2) | +++ | +++ | + |
| PATIENT 5 (1) | ++ | +/- | - |
| PATIENT 5 (2) | ++ | +/- | - |
| PATIENT 5 (3) | ++ | + | - |
| PATIENT 6 | +++ | ++ | - |
| PATIENT 7 | + | +/- | - |
| PATIENT 8 | - | +/- | - |
| PATIENT 9 | +/- | +/- | - |
| PATIENT 10 | + | + | - |
| PATIENT 11 | ++ | + | - |
| PATIENT 12 | +++ | + | - |
| SALAD 1 | ++ | +++ | - |
| SALAD 3 | +++ | + | - |
| SALAD 4 | ++ | +++ | - |

No significant difference was found between the environmental and clinical samples: most of them exhibited high to moderate protease and lipase activity and only two clinical strains had some kind of elastase activity. Only three of the four environmental strains could be tested as one of them (salad 2) proved unable to grow at 37°C. This could be due to the possession of a 'temperature-regulated suicide system' found in environmental strains that makes them unable to survive at body temperatures (Alavi *et al.*, 2014).

4.3. Pathogenicity of *S. maltophilia*

4.3.1. Myeloperoxidase (MPO) and Neutrophil Elastase (NE) activity

Since previous studies found a relationship between *P. aeruginosa* infection and MPO and NE activity, those two inflammatory markers were chosen to test *S. maltophilia* for pathogenicity (Chalmers *et al.*, 2012).

Results (provided by Dr. Pallavi Bedi) from sputum samples that had grown mixed normal flora in patients with bronchiectasis and not chronically colonised were used as negative controls (n=7) since inflammatory markers in their sputum should solely be due to the bronchiectasis condition itself.

Since these assays require spun sputum samples which are only stored when a sufficient amount is produced by the patient to cover the preliminary quantitative and qualitative microbiological routine, these were not systematically available for every *S. maltophilia* episode and it was quite challenging to gather a decent amount of them. Only 10 spun sputum samples were found. These were matched in gender and age with the negative controls for an accurate comparison (annexe 5).

MPO levels in sputum with MNF vs *S. maltophilia* (SM)

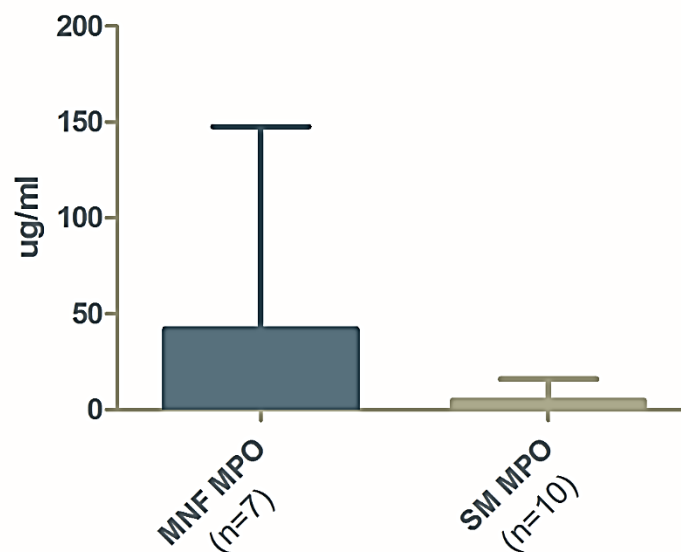


Figure 4.11 Comparison of MPO levels in sputum with MNF and in sputum with *S. maltophilia* (SM): the difference between both is not significant ($p = 0.3795$).

As shown above in Figure 4.11, there was no significant difference ($p\text{-value} > 0.05$) between the levels of MPO in the sputum samples containing MNF and in the ones containing *S. maltophilia*. On the other hand, NE levels were significantly higher ($*p\text{-value} < 0.01$) in sputum samples containing *S. maltophilia* than in the ones containing MNF (Fig. 4.12).

NE levels in sputum with MNF vs *S. maltophilia* (SM)

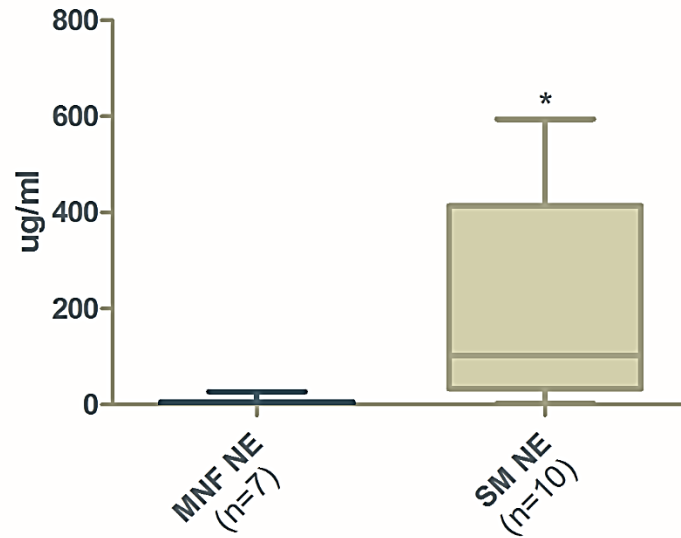


Figure 4.12 Comparison of NE levels in sputum with MNF and in sputum with *S. maltophilia* (SM): the difference between both is highly significant (*p-value = 0.003).

5. Discussion

5.1. Antibiotic treatment as a risk factor for *S. maltophilia*

The results from this study support the hypothesis that *S. maltophilia* tends to appear in patients that have been exposed to strong or longer antibiotic regimes and thus provides evidence to suggest that aggressive antibiotic treatments could be a risk factor for *S. maltophilia* infection by promoting its establishment in bronchiectasis patients' lung.

In fact, among the general bronchiectasis cohort, most cases of *S. maltophilia* occurred in patients from the higher spectra of severity, thus having been subjected to more antibiotic courses due to exacerbations and progression of the disease. Similarly, the disparity between its incidence in CF –where patients are subjected to antibiotic treatment since a younger age and thus for a longer period of time– and bronchiectasis further supports that hypothesis.

Nevertheless, it is important to highlight that the low rates of incidence of *S. maltophilia* in bronchiectasis might also be due to poor diagnosis and thus be under- or mis-reported by hospital laboratories. In fact, through this research project, a few occasions of discrepancies between the clinical and research laboratories was observed. This could be due to an uneven spread of the bacteria in the sputum which leads to an incomplete diagnosis as it gets split between laboratories or sometimes patients provide two different pots of sputum, one from the morning and a second one that they produce during the clinic and which could be different to each other. It could also be due to non-specific identification from clinical labs which could be solved by the use of selective media (M Denton *et al.*, 2000), processing sputum quantitatively and by raising awareness for the bacterium among the staff as it might also get neglected and labelled as MNF, considered non-pathogenic.

5.2. Need for sensitivity guidelines specific to *S. maltophilia*

An alarming finding during this study was the lack of guidelines for antibiotic sensitivity specific to *S. maltophilia*: these were only available for TMP-SXT and minocycline (Hombach, Bloemberg and Böttger, 2012). The research in treatment of the bacterium is hugely deficient and clinicians can currently only resort to their best judgement when deciding whether and how to treat *S. maltophilia*.

The different resistance patterns observed between CF and bronchiectasis strains also illustrated how current treatment is probably shaped based on studies from cystic fibrosis studies since the preferred treatment for *S. maltophilia* used in clinics and wards is TMP-SXT (Hand *et al.*, 2016)

However, as shown in this study, despite being the most efficient antibiotic, at least *in vitro* for CF strains, other antibiotics displayed greater sensitivity among bronchiectasis strains, which should be further investigated *in vivo*, especially following the increasing incidence of TMP-SXT resistance in *S. maltophilia* (Toleman *et al.*, 2007).

5.3. *S. maltophilia* as a potential ‘artificial commensal’

An important observation highlighted by Amin and Waters (2016) in their review that looked at *S. maltophilia* in CF is the lack of guidelines for treatment of *S. maltophilia* and especially of studies that compare its treatment to its *non-treatment*.

Being an intrinsically multidrug resistant organism, treatment of *S. maltophilia* implies the use of last line antibiotics (Milne and Gould, 2012) which can have serious side-effects and greatly affect the lung microbiome (Boyton *et al.*, 2013). If *S. maltophilia* was proven *not* to be implicated in disease progression, the idea of avoiding its treatment at first isolation should be considered.

Bronchiectasis patients’ normal flora most likely looks very different to that of healthy patients and, especially in the most advanced cases, has been severely depleted by aggressive antimicrobial treatments (Boyton *et al.*, 2013) to remove pathogens causing exacerbations. As a consequence, they end up becoming colonised or infected with increasingly resistant strains or species which become increasingly difficult and, eventually, almost impossible to treat. The empty niches left behind after antibiotic regimes are propitious for opportunistic pathogens infections and this can lead to an additional vicious cycle in bronchiectasis.

Therefore, proven that *S. maltophilia* is non-pathogenic or specific strains of *S. maltophilia* are non-pathogenic, these could be used as ‘artificial commensals’ to fill in the niches and prevent other more harmful bacteria from integrating the ecosystem, creating thereby a kind of ‘artificial commensal ecosystem’.

Understandably, the main fear remains the potential risk for horizontal gene transfer of its wide range resistance to other pathogenic bacteria which is presumably the main reason for the tendency to treat patients at first isolation of *S. maltophilia* (Crossman *et al.*, 2008). However, extensive studies on the resistance mechanisms have shown that most antibiotic resistance genes in *S. maltophilia* are not related to mobile genetic elements (Ryan *et al.*, 2009). Further studies and a clinical trial looking at the ‘non-treatment’ of *S. maltophilia* should be carefully monitored and analysed before making any conclusion but are definitely worth considering.

5.4. Implication of *S. maltophilia* in disease progression

A few observations from this study seem to suggest that *S. maltophilia* behaves quite similarly to the known pathogen *P. aeruginosa*, consistent with its initial classification as a *Pseudomonas* species.

First, lung function and serological variables compared from 14-day IV-antibiotic courses where either *S. maltophilia*, *P. aeruginosa* and *H. influenzae* had been initially isolated, suggested that *S. maltophilia* was having an effect more similar to *P. aeruginosa* than *H. influenzae* in bronchiectasis patients. However, from the subsequent analyses, this could be rather attributed to a more efficient clearing of *H. influenzae* following IV-antibiotic treatment compared to *P. aeruginosa* and especially *S. maltophilia* which seems to be unaffected and even *promoted* following IV antibiotic treatment.

On the other hand, inflammatory markers experiments looking at MPO and NE levels in sputum revealed a significant difference of elastase levels in sputum with *S. maltophilia* compared to that in sputum carrying MNF. This is also a known property of *P. aeruginosa* which elicits elastase activity in sputum as well as MPO (Chalmers *et al.*, 2012) and could be a sign of pathogenicity.

However, there is insufficient evidence to confidently support or refute the pathogenicity of *S. maltophilia* and further experiments are needed: there seems to be no simple answer to this question but to do so, establishing the chronology between infection and disease progression will be essential. An *in vitro* experiment that would provide reliable evidence is to perform ELISA experiments looking at IL-8 production of lung cells upon infection with *S. maltophilia* and compare them to that of uninfected cells and cells infected with a known pathogen such as *P. aeruginosa*, which is part of the future work planned.

5.5. Environmental versus clinical

The results from the experiments that aimed at comparing clinical strains with environmental ones suggest that antimicrobial susceptibility and virulence factors might not be very determining. Further genomics analysis might be required to establish more subtle differences and look at specific genes such as RNDs to potentially link certain types of strains with particular clinical outcomes of *S. maltophilia* infection (Crossman *et al.*, 2008; Youenou *et al.*, 2015).

5.6. Future work

As mentioned above, experiments are currently on-going to provide further evidence regarding the pathogenicity of *S. maltophilia*. These would look *in vitro* at the inflammatory response of healthy cells upon infection of *S. maltophilia* which would help establish the chronology in the disease progression, removing any underlying inflammation purely due to bronchiectasis itself.

However, in the scenario of evidence heading towards the refutation of its pathogenicity, controlled studies assessing its non-treatment should be considered. Alternatively, if its pathogenicity is confirmed, it will raise the urgent need for trials and disease-specific guidelines for antimicrobial treatment.

Finally, an interesting aspect that arose following this study, is the potential implication of IV-antibiotic treatment as a risk factor for *S. maltophilia* infection. From the data analyses performed on patients on the IV-treated sub-group, IV-antibiotic therapy seemed to *promote* isolation of *S. maltophilia*. This could be either due to the antibiotics themselves clearing commensals and thus leaving empty niches behind as it was previously discussed, or it could be due to the IV mode of delivery itself: as an indwelling device, it could be a route of infection but in such case, one would expect accompanying skin infection at the level of the port which was not observed in our cohort. This definitely represents an interesting observation and deserves further examination as to whether nebulised administration of antibiotics is more efficient than intravenous.

5.7. Limitations

The main limitation of this study is the sample size. With a limited time-frame attempting to collect samples directly from patients wasn't an easy task, especially as it seems like *S. maltophilia* being originally an environmental organism might be season dependent: samples took a while to reach a significant number sufficient to do any experiments with them.

Similarly, isolation of *S. maltophilia* from environmental sources was low and a higher number of samples would have probably allowed for stronger evidence and better data.

Furthermore, using retrospective data also meant being confronted with severely incomplete databases. For further studies, establishment of generic checklists of data to be systematically collected at clinic visits would significantly benefit the reliability of the database and facilitate retrospective research studies.

5.8. Concluding remarks

The work presented here aimed at providing some characterisation of *S. maltophilia* in bronchiectasis and raise awareness of the importance of its accurate diagnosis among bronchiectasis patients as it seems that it has long been neglected and overlooked but appears to have an increasing importance in bronchiectasis.

This work also highlights the need for a clear confirmation of the pathogenicity of the species in order to provide coherent guidelines for its subsequent treatment or non-treatment after isolation from patients' sputum.

In the scenario of evidence leading towards the confirmation that *S. maltophilia* does not contribute to a deterioration of the patients' lung condition, this study proposes a somewhat controversial use of *S. maltophilia* as an 'artificial commensal', using its intrinsic resistance enabling it to occupy the patients' lung after aggressive antimicrobial treatment to prevent further pathogens from invading the empty niches.

6. Acknowledgements

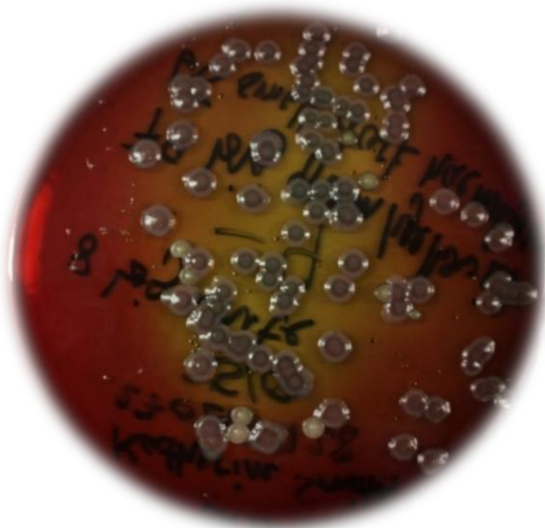
I would like to thank Prof. Adam Hill for having me in his lab, providing me with lab space and supervising me through the project. I also want to thank Dr. Catherine Doherty for helping me and teaching me all the techniques related to the microbiology part of the project as well as for her feedback and for providing me with the CF strains used in my project. Similarly, I am grateful to Dr. Pallavi Bedi for her help with the molecular biology side of the project, as well as for providing me with the data from the negative controls regarding the MPO and NE assays. I am also very grateful to Beatriz Herrero for her valuable help with the statistics part of the project and data analysis and especially for her patience sharing her expertise in the field. Thanks as well to the respiratory nurse specialist Kim Turnbull for providing me with the IV and nebulised antibiotics patients database. Last but not least, I want to give a special thanks to the two research nurses, Andrea Clarke and Samantha Donaldson for their valuable help getting the necessary sputum samples to get the project started as well as for their constant support through the ups and downs of this challenging year. I also thank Dr. Kim Picozzi, the program director for her availability and guidance throughout the year.

In addition, I will always be immensely grateful to my family, my parents and my twin sister for their unconditional love and support from the distance and their pride for every step I achieve towards my professional aspirations, each of which I owe to them as I would have never gotten there without them.

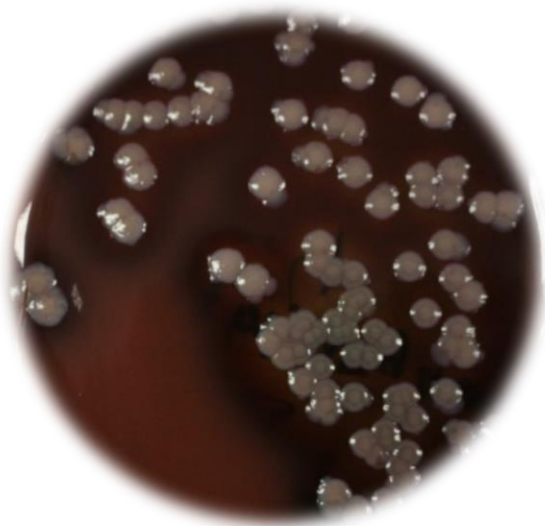
Finally, I want to make a special dedication of this thesis to all the bronchiectasis patients from ward 204 and the respiratory clinics for their input since nothing would have been possible without their contribution. A special thanks to the wonderful Bronchiectasis Support Group set up by the bronchiectasis patients themselves which welcomed me very warmly since day one and kept me motivated to do my best, they are a great example of public engagement linking researchers to the general public.

7. Annexes

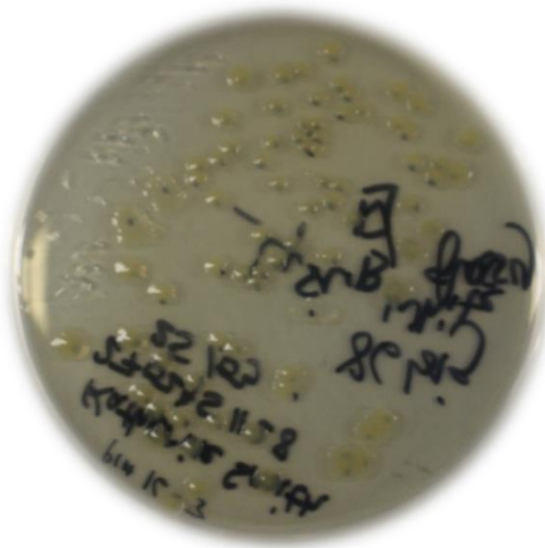
Annexe 1: Appearance of *S. maltophilia* on different selective media used for its isolation and identification



S. maltophilia appearance on blood agar (BA): centre appears darker and outer ring is clearer with an olive-like colour.



S. maltophilia appearance on chocolate blood agar (CBA): big opaque colonies with a line showing centre and outer ring, brownish colour.

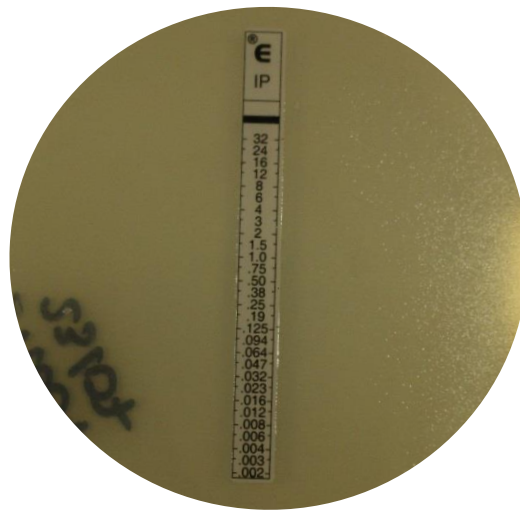


S. maltophilia appearance on *Pseudomonas* isolation agar (PIA): centre appears darker and outer ring is clearer with a glossy transparent yellowish colour.

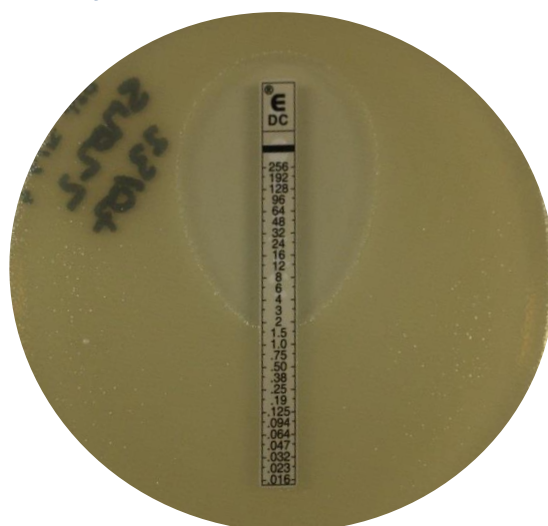
Annexe 2: Figures of *S. maltophilia* strains tested for antimicrobial sensitivity using Etest strips of different antibiotics (Tobramycin, Imipenem, Doxycycline, TMP-SXT, Meropenem)



Tobramycin (TM) Etest of a *S. maltophilia* strain: shows MIC of 0.5 µg/ml



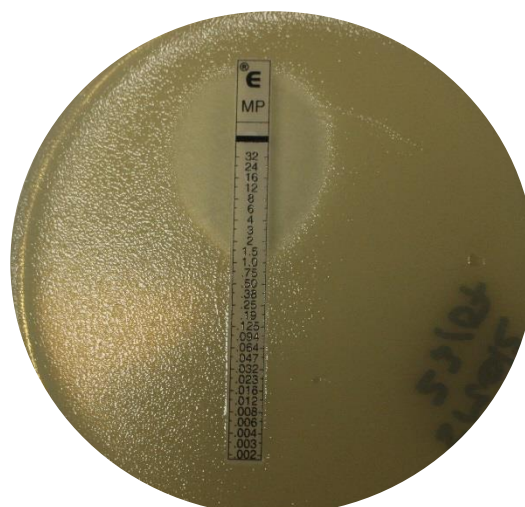
Imipenem (IP) Etest of a *S. maltophilia* strain: shows full resistance to Imipenem (MIC = 32 µg/ml)



Doxycycline (DC) Etest of a *S. maltophilia* strain: shows MIC of 2 µg/ml



TMP-SXT (TS) Etest of a *S. maltophilia* strain: shows MIC of 0.064 µg/ml



Meropenem (MP) Etest of a *S. maltophilia* strain: shows MIC of 1 µg/ml

Annexe 3: Complementary data on matched patients for the *S. maltophilia*, *P.aeruginosa* and *H. influenzae* IV subgroup variables comparison

| | <i>S. maltophilia</i> (n=11) | <i>P. aeruginosa</i> (n=22) | <i>H. influenzae</i> (n=22) | p-value |
|-------------------|---------------------------------|--------------------------------|--------------------------------|-------------|
| Sex (Male) | 64% | 77% | 36% | 0.03 |
| Age | 67 [63 – 74] | 72 [64.5 – 73.25] | 68 [63 – 71] | 0.20 |
| BSI | 8 [8 – 10] | 11 [9.75 – 15] | 11 [5.75 – 12] | 0.03 |

Annexe 4: Complementary data on library of environmental and bronchiectasis *S. maltophilia* isolates

| IDENTIFICATION | DATE OF COLLECTION | ADDITIONAL INFORMATION |
|----------------------|--------------------|---|
| PATIENT 1 (1) | 11/02/2015 | Sample isolated prior to 14-day IV-antibiotic course (at start of exacerbation) |
| PATIENT 1 (2) | 15/02/2015 | Sample isolated at day-14 following IV-antibiotic course (at end of exacerbation) |
| PATIENT 2 (1) | 24/03/2014 | Sample isolated at the start of an exacerbation |
| PATIENT 2 (2) | 07/04/2014 | Sample isolated at the end of an exacerbation with antibiotic therapy |
| PATIENT 3 (1) | 02/04/2014 | Sample isolated from a stable sputum production |
| PATIENT 3 (2) | 08/03/2016 | Sample isolated from a stable sputum production |
| PATIENT 4 (1) | 19/02/2016 | Sample isolated at day-14 of a 14-day IV meropenem course |
| PATIENT 4 (2) | 25/02/2016 | Sample isolated at day-21 of a 14-day IV meropenem course |
| PATIENT 5 (1) | 17/03/2016 | Sample isolated at day-10 of a 8-day IV meropenem course |
| PATIENT 5 (2) | 21/04/2016 | Sample isolated at day-14 of a 8-day IV meropenem course |
| PATIENT 5 (3) | 28/04/2016 | Sample isolated at day-21 of a 8-day IV meropenem course |
| PATIENT 6 | 12/07/2015 | Sample isolated at day-10 of a 14-day IV meropenem course |
| PATIENT 7 | 07/08/2014 | Sample isolated at day-14 of a 8-day IV meropenem course |
| PATIENT 8 | 24/03/2014 | Sample isolated from a stable sputum production |
| PATIENT 9 | 25/03/2014 | Sample isolated from a stable sputum production |
| PATIENT 10 | 12/02/2016 | Sample isolated at day-21 of IV 14-day meropenem course |
| PATIENT 11 | 17/03/2016 | Sample isolated from a sputum produced by an inpatient with an exacerbation |
| PATIENT 12 | 17/03/2016 | Sample isolated from a sputum produced by an inpatient with an exacerbation |
| SALAD 1 | 09/02/2016 | Sample isolated from a green leaves salad sample |
| SALAD 2 | 09/02/2016 | Sample isolated from a spinach sample |
| SALAD 3 | 24/02/2016 | Sample isolated from a mixed leaves salad sample |
| SALAD 4 | 24/02/2016 | Sample isolated from a watercress sample |

Annexe 5: Complementary data on matched patients for the MNF/*S.maltophilia* comparison of MPO and NE levels

| | MNF (n=7) | <i>S. maltophilia</i> (n=10) | p-value |
|-------------------|----------------------|---|------------------|
| Sex (Male) | 43% | 50% | 1.00 |
| Age | 69 [53 – 78] | 74 [65.5 – 80] | 0.27 |
| BSI | 4 [2 – 4] | 11 [6 – 11.25] | <0.001 |

8. Abbreviations

BA – Blood agar

BHI – Brain Heart Infusion

BSA – Bovine Serum Albumin

CBA – Chocolate Blood agar

CF – Cystic fibrosis

DST [agar] – Diagnostic Sensitivity Test [agar]

DTT – Dithiothreitol

ELISA – Enzyme-linked immunosorbent assay

HI – *Haemophilus influenzae*

IL-8 – Interleukin 8

IV [antibiotics] – Intravenous [antibiotics]

MIC – Minimum inhibitory concentration

MNF – Mixed Normal Flora

MPO – Myeloperoxidase

NA – Nutrient agar

NB – Nutrient broth

NE – Neutrophil elastase

OD – Optical density

PA – *Pseudomonas aeruginosa*

PFGE – Pulsed-Field Gel Electrophoresis

PIA – *Pseudomonas* Isolation agar

QoL – Quality of Life

SM – *Stenotrophomonas maltophilia*

TBE [buffer] – Tris Borate EDTA [buffer]

VIA – Vancomycin, Imipenem and Amphoterecin B

YE – Yeast Extract

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